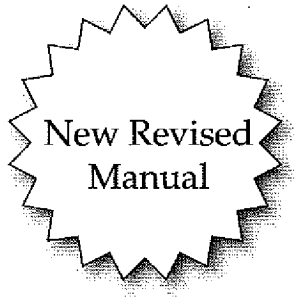
Exhibit M

invitrogen™

SuperScript® Full Length cDNA Library Construction Kit

High-quality cap antibody selected full length cDNA libraries without the use of restriction enzyme cloning techniques

Catalog no. A11181



Rev. Date: 6 November 2009
Part no. A11406

MAN0001711

User Manual

Table of Contents

Kit Contents and Storage

Shipping/Storage

The Superscript® Full Length cDNA Library Construction Kit is shipped on dry ice. Upon receipt, store the components as detailed below. All components are guaranteed for six months if stored properly.

Item	Storage
Superscript® Full Length cDNA Library Construction Kit Cap Antibody Module	+4°C
Superscript® Full Length cDNA Library Construction Kit Core Module	–20°C
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	–80°C

Number of Reactions

The Superscript® Full Length cDNA Library Construction Kit provides enough reagents to construct five cDNA libraries. While some reagents are supplied in excess, you may need additional reagents and materials if you wish to perform more than 5 reactions.

Components for Cap Antibody Module

The components for cDNA library construction are listed below. Store all components at 4°C.

Item	Composition	Amount
Cap-antibody Beads	10 mg/mL; 100 µg/Ab/mg bead	1.65 mL
Spin Columns	6 columns per bag	3 bags
TEN Buffer	10 mM Tris-HCl, pH 7.5 0.1 mM EDTA 25 mM NaCl	40 mL
TENG Buffer	10 mM Tris-HCl, pH 7.5 0.1 mM EDTA 25 mM NaCl 1 mM GDP	2 mL
5 M NaOH	5 M NaOH	100 µL
cDNA Size Fractionation Column	3 columns per box	2 boxes
1 M Tris-HCl, pH 7.0	---	1 mL
Binding Buffer	---	5 mL
Wash Buffer	---	2.5 mL

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Kit Contents and Storage, continued

Components for Core Module

The components for cDNA library construction are listed below. Store all components at –20°C.

Item	Composition	Amount
Yeast tRNA	10µg/µl	20 µL
DEPC-treated Water	Sterile, DEPC-treated water	2 × 2 mL
10 mM (each) dNTP	10 mM dATP 10 mM dGTP 10 mM dCTP 10 mM dTTP in 1 mM Tris-HCl, pH 7.5	2 × 20 µL
5X First Strand Buffer	250 mM Tris-HCl, pH 8.3 375 mM KCl 15 mM $MgCl_2$	1 mL
7.5 M Ammonium Acetate	7.5 M NH_4OAc	2 × 1 mL
0.1 M Dithiothreitol (DTT)	in DEPC-treated water	100 µL
SuperScript® III Reverse Transcriptase	200 U/µL in: 20 mM Tris-HCl, pH 7.5 0.1 mM EDTA 100 mM NaCl 0.01% NP-40 (v/v) 1 mM DTT 50% Glycerol (v/v)	30 µL
10X RNase I buffer	---	200 µL
RNase I	100 U/µL	12 µL
UltraPure™ Glycogen	20 µg/µL in RNase-free water	50 µL
T4 DNA Ligase	1 U/µL in: 100 mM Potassium Phosphate, pH 6.5 10 mM β-mercaptoethanol 50% Glycerol (v/v)	50 µL
pDONR™ 222 Vector	150 ng/µL vector in: 10 mM Tris-HCl 1 mM EDTA, pH 8.0	15 µL
BP Clonase™ II Enzyme Mix	Proprietary	40 µL
Proteinase K	2 µg/µL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% Glycerol (v/v)	40 µL

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Kit Contents and Storage, continued

Components for Core Module, continued

Item	Composition	Amount
*att*B2-dT22VN Primer	1.5 µg/µL in DEPC-treated water	15 µL
*att*B1 Adapter Mix	0.5 µg of adapters in: 10 mM Tris-HCl, pH 7.5 1 mM EDTA 0.1 M NaCl	25 µL
5X Adapter Buffer	330 mM Tris-HCl, pH 7.6 50 mM $MgCl_2$ 5 mM ATP	70 µL
5′ Primer	0.1 µg/µL of adapters in: 10 mM Tris-HCl, pH 7.5 1 mM EDTA 0.1 M NaCl	10 µL

*att*B2-dT22VN Primer Sequence

The *att*B2-dT22VN Primer is biotinylated to block blunt-end ligation of the *att*B1 Adapter to the 3′ end of the cDNA during the adapter ligation step. The primer sequence is provided below with the *att*B2 sequence in bold.

5′-Biotin-GGGG**ACAACTTTGTACAAGAAAGTTGGGT**GAATTC(T)$_{22}$VN-3′

*att*B1 Adapter Mix Sequences

The double-stranded adapter is made by denaturation and slow annealing of the two oligonucleotides in annealing buffer. The sequence is provided below with the *att*B1 sequence in bold.

5′-TCGTCGGGG**ACAACTTTGTACAAAAAAGTTGGCGGCCGCC**-3′
3′-CCCC**TGTTGAAACATGTTTTTTCAACCGCCGGCGG**p-5′

5′ Primer Sequence

The 5′ Primer is used for synthesizing the second strand of cDNA. The primer sequence is provided below.

5′-TCGTCGGGGACAACTTTGTACAAAAAAGTTGGCGGCCGCC-3′

continued on next page

Kit Contents and Storage, continued

DH10B™ T1 Phage Resistant Cells

Three boxes of ElectroMAX™ DH10B™ T1 Phage Resistant Cells are provided with the kit. Transformation efficiency is >1 × 10^{10} cfu/µg DNA. Each box includes the following items. Store at –80°C.

Item	Composition	Amount
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	---	5 × 100 µL/box (3 boxes)
pUC19 Control DNA	10 pg/µL in: 5 mM Tris-HCl 0.5 mM EDTA, pH 8	50 µL
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glucose	2 × 6 mL

Genotype of DH10B™ T1 Phage Resistant Cells

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G *ton*A

cDNA Size Fractionation Columns

Two boxes containing three disposable columns each are provided with the kit for a total of six columns. Each column contains 1 mL of Sephacryl® S-500 HR prepacked in 20% ethanol. Store columns at +4°C.

Required Products Not Supplied in Kit

The Superscript® Full Length cDNA Library Construction Kit requires the following additional products to be purchased separately before use of the kit for library construction.

Item	Catalog no.
Platinum® *Taq* DNA Polymerase High Fidelity	11304-011
Magna-Sep™ Magnetic Particle Separator	K1585-01

Introduction

About the Kit

Kit Usage

The Superscript® Full Length cDNA Library Construction Kit is designed to generate high-quality cDNA libraries containing full length transcriptome sequences. This novel technology combines the performance of SuperScript® III Reverse Transcriptase with cap antibody selection to provide a library enriched for full length clones, while the use of Gateway® Technology allows library construction to be performed without traditional restriction enzyme cloning methods.

Single-stranded mRNA is converted into double stranded cDNA containing *att*B sequences on each end. PCR amplification is not required, thus avoiding bias that may be introduced by amplification. Through site-specific recombination, *att*B-flanked cDNA is cloned directly into an *att*P-containing donor vector without the use of restriction digestion or ligation.

The resulting Gateway® entry cDNA library can be used for various downstream applications including library amplification, library screening, mapping the transcriptional start site of your gene, identification of novel 5′ UTR sequence, and next-generation sequencing. Clones or the entire library can also be transferred into Gateway® destination vectors for gene expression and functional analysis. For more information on the Gateway® Technology, see page 3.

Features of the Superscript® Full Length cDNA Library Construction Kit

- SuperScript® III reverse transcriptase for efficient conversion of mRNA into cDNA
- Biotinylated *att*B2-dT22VN Primer for poly(A) mRNA binding and incorporation of the *att*B2 sequence to the 3′ end of cDNA
- Cap antibody selection and RNase I treatment to enrich for full length cDNA clones containing sequences beginning with the transcriptional start site
- *att*B1 Adapter for ligation of the *att*B1 sequence to the 5′ end of the first strand cDNA
- *att*P-containing vector (pDONR™ 222) for recombination with *att*B-flanked cDNA to produce an entry library through the Gateway® BP recombination reaction (see pages 52-53 for a map and list of features)

Advantages of the Superscript® Full Length cDNA Library Construction Kit

- Produces a cDNA library representing full length transcripts
- Produces a higher number of primary clones compared to standard cDNA library construction methods (Ohara and Temple, 2001) through efficient recombinational cloning of cDNA into a donor vector
- Reduces the number of chimeric clones and reduces size bias with respect to standard cDNA library construction methods (Ohara and Temple, 2001)
- Eliminates use of restriction enzyme digestion and ligation allowing cloning of undigested cDNA
- Enables highly efficient transfer of your cDNA library into multiple destination vectors for protein expression and functional analysis

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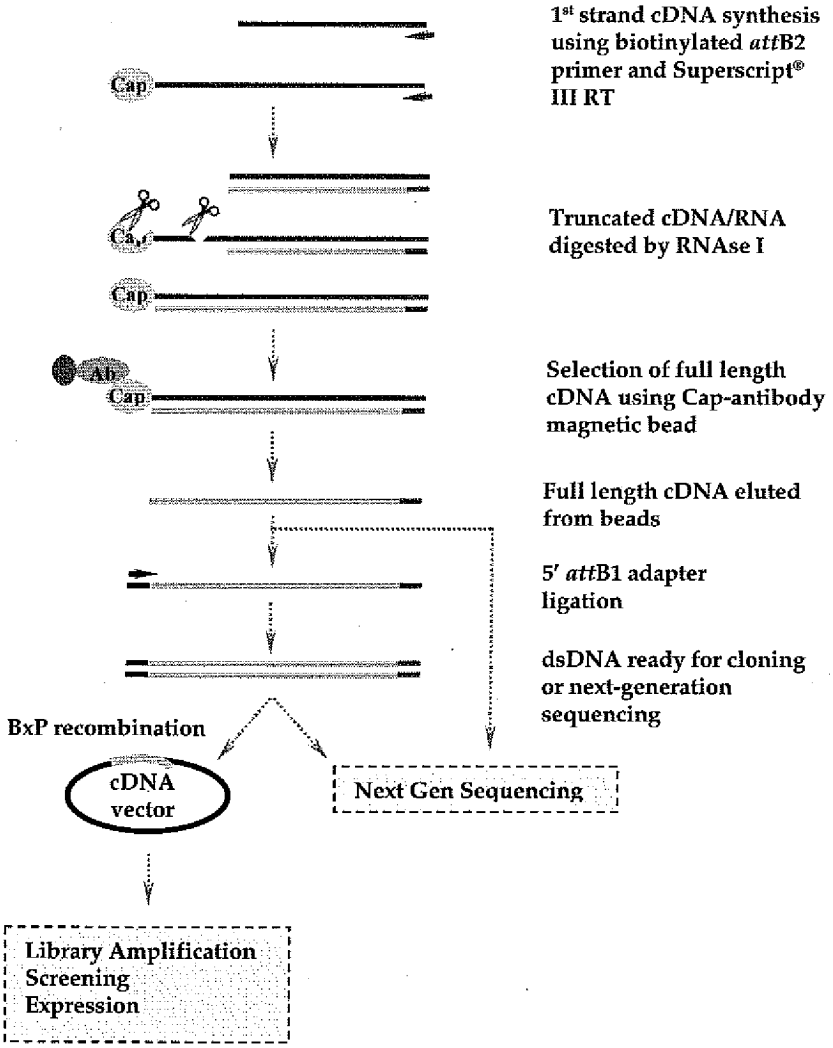
About the Kit, continued

Summary of cDNA Synthesis Procedure

The following diagram summarizes the cDNA synthesis process of the Superscript® Full Length cDNA Library Construction Kit.



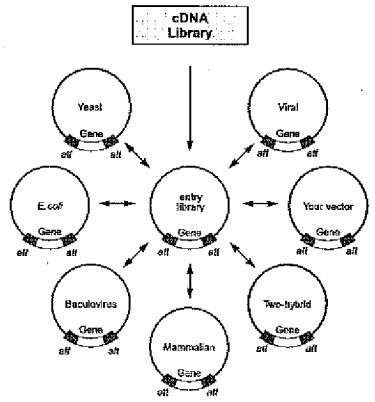
The Gateway® Technology

The Basis of Gateway®

The Gateway® Technology is based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the *E. coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1992). In the Gateway® Technology, the components of the lambda recombination system are modified to improve the specificity and efficiency of the system (Bushman *et al.*, 1985). This section provides a brief overview of the Gateway® Technology. For detailed information, refer to the Gateway® Technology manual. This manual is available from our website (www.invitrogen.com) or by contacting Technical Support (page 54).

The Gateway® Technology

Gateway® is a universal cloning technology based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989). The Gateway® Technology provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression. For more information on the Gateway® Technology, see the next page.



Recombination Components

Lambda-based recombination involves two major components:

- The DNA recombination sequences (*att* sites) **and**
- The proteins that mediate the recombination reaction (*i.e.* Clonase™ enzyme mix)

Characteristics of Recombination Reactions

Lambda integration into the *E. coli* chromosome occurs via intermolecular DNA recombination that is mediated by a mixture of lambda and *E. coli*-encoded recombination proteins (*i.e.* Clonase™ enzyme mix). The hallmarks of lambda recombination are:

- Recombination occurs between specific (*att*) sites on the interacting DNA molecules.
- Recombination is conservative (*i.e.* there is no net gain or loss of nucleotides) and does not require DNA synthesis. The DNA segments flanking the recombination sites are switched, such that after recombination, the *att* sites are hybrid sequences comprised of sequences donated by each parental vector (*e.g.*, *att*L sites are comprised of sequences from *att*B and *att*P sites).
- Strand exchange occurs within a core region that is common to all *att* sites (see next page).

See references (Landy, 1989; Ptashne, 1992) for more information about lambda recombination,.

continued on next page

The Gateway® Technology, continued

att Sites

Lambda recombination occurs between site-specific attachment (*att*) sites: *att*B on the *E. coli* chromosome and *att*P on the lambda chromosome. The *att* sites serve as the binding site for recombination proteins and have been well characterized (Weisberg and Landy, 1983). Upon lambda integration, recombination occurs between *att*B and *att*P sites to give rise to *att*L and *att*R sites. The actual crossover occurs between homologous 15 bp core regions on the two sites, but surrounding sequences are required as they contain the binding sites for the recombination proteins (Landy, 1989).

In the Superscript® Full Length cDNA Library Construction Kit, the wild-type *att*B sites encoded by the *att*B1 Adapter and *att*B2-dT22VN Primer and the wild-type *att*P1 and *att*P2 sites encoded by pDONR™ 222 are modified to improve the efficiency and specificity of the Gateway® BP recombination reaction.

*ccd*B Gene

The presence of the *ccd*B gene in pDONR™ 222 allows negative selection of the donor vector in *E. coli* following recombination and transformation. The CcdB protein interferes with *E. coli* DNA gyrase (Bernard and Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (*e.g.* DH5α™, TOP10). When recombination occurs between pDONR™ 222 and the *att*B-flanked cDNA, the *ccd*B gene is replaced by the cDNA insert. Cells that take up nonrecombined pDONR™ 222 carrying the *ccd*B gene or by-product molecules retaining the *ccd*B gene will fail to grow. This allows high-efficiency recovery of the desired clones.

Gateway® Recombination Reactions

Two recombination reactions constitute the basis of the Gateway® Technology. By using the Superscript® Full Length cDNA Library Construction Kit, you can take advantage of these two reactions to clone and shuttle your cDNA library into a destination vector of choice.

BP Reaction: Facilitates recombination of *att*B-flanked cDNA with an *att*P-containing vector (pDONR™ 222) to create an *att*L-containing entry library (see diagram below). This reaction is catalyzed by BP Clonase™ II enzyme mix.

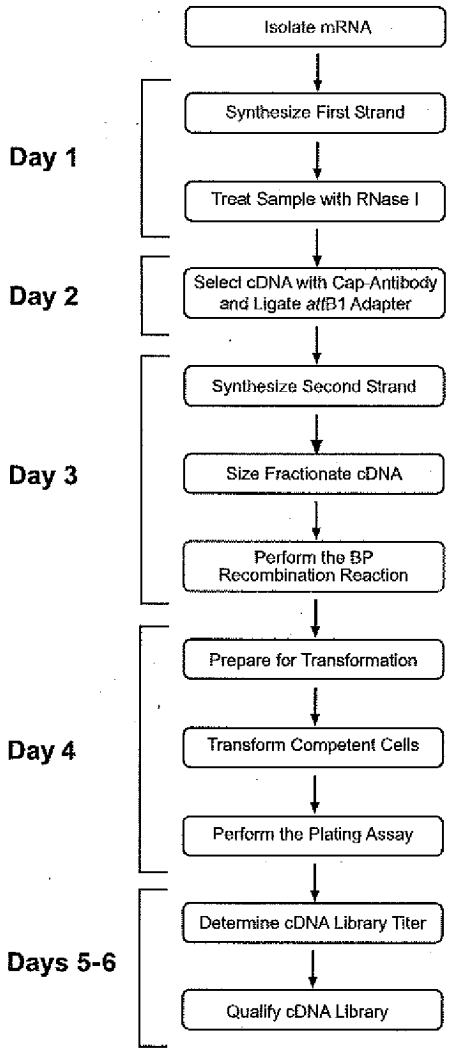
LR Reaction: Facilitates recombination of an *att*L entry clone or entry library with an *att*R substrate (destination vector) to create an *att*B-containing expression clone or expression library (see diagram below). This reaction is catalyzed by LR Clonase™ or LR Clonase™ II enzyme mix.

Experimental Timeline

Introduction

The Superscript® Full Length cDNA Library Construction Kit is designed to produce an entry library from your starting mRNA within three days. It will take an additional two days to determine the titer and quality of the cDNA library. Note that this manual is organized according to the recommended timeline below. If you are not following this timeline, be sure to plan ahead for convenient stopping points (see below for more information).

Recommended Timeline



Optional Stopping Points

If you cannot follow the recommended timeline, you may stop the procedure during any ethanol precipitation step. When stopping at these points, always store the cDNA as an **uncentrifuged** ethanol precipitate at –20°C to maximize cDNA stability.

Experimental Overview

Introduction

The experimental steps necessary to synthesize *att*B-flanked cDNA and to generate an entry library are outlined below. Once you have isolated your mRNA (page 7), you will need a minimum of 3 days to construct a cDNA library. For more details on each step, refer to the indicated pages for your specific method.

Day	Step	Action	Page
1	1	Synthesize the first strand of cDNA from your isolated mRNA using the *att*B2-dT22VN Primer and SuperScript® III RT.	10
	2	Treat first strand cDNA with RNase I	14
2	1	Select full length cDNA with Cap-antibody beads	17
	2	Ligate the *att*B1 adapter to the 5′ end of your cDNA.	20
3	1	Synthesize the second strand of cDNA using the first strand cDNA as a template.	22
	2	Size fractionate the cDNA by column chromatography to remove excess primers, adapters, and small cDNA.	24
	3	Perform the BP recombination reaction between the *att*B-flanked cDNA and pDONR™ 222.	28
4	1	Transform the BP reactions into ElectroMAX™ DH10B™ T1 Phage Resistant cells by electroporation. Add freezing media to transformed cells to get final cDNA library.	31
	2	Perform the plating assay to determine the cDNA library titer.	35
5–6	1	Calculate the cDNA library titer using the results from the plating assay.	37
	2	Inoculate 24 positive transformants from the plating assay. Determine average insert size and percent recombinants by restriction analysis.	38
	3	Sequence entry clones to verify presence of cDNA insert, if desired.	40

Methods

Isolating mRNA

Introduction

You will need to isolate high-quality mRNA using a method of choice prior to using this kit. Follow the guidelines provided below to avoid RNase contamination.

Aerosol-resistant pipette tips are recommended for all procedures. See below for general recommendations for handling mRNA.

General Handling of mRNA

When working with mRNA:

- Use disposable, individually wrapped, sterile plasticware
- Use only sterile, RNase-free pipette tips and RNase-free microcentrifuge tubes
- Wear latex gloves while handling all reagents and mRNA samples to prevent RNase contamination from the surface of the skin
- Always use proper microbiological aseptic technique when working with mRNA
- You may use RNase Away™ Reagent, or RnaseZap® available from Invitrogen (see page 45 for ordering information), to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989).

mRNA Isolation

mRNA can be isolated from tissue, cells, or total RNA using the method of choice. We recommend isolating mRNA using the FastTrack™ MAG Micro mRNA Isolation Kit or the FastTrack™ MAG Maxi mRNA Isolation Kit available from Invitrogen (see page 45 for ordering information).

Generally, 5 to 10 µg of mRNA is sufficient to construct a cDNA library containing 10^6 to 10^7 primary clones in *E. coli*. Using less mRNA results in fewer primary clones.

Resuspend isolated mRNA in DEPC-treated water and check the quality of your preparation (see next page). Store your mRNA preparation at –80°C. We recommend aliquoting your mRNA into multiple tubes to reduce the number of freeze/thaw cycles.

Important

To assure the creation of a cDNA library with adequate representation of primary clones when using the Superscript® Full Length cDNA Library Construction Kit, it is recommended to proceed with library creation only when you have the recommended amount of starting mRNA.

To ensure success, use the highest quality mRNA possible. Check the quantity, integrity, and purity of your mRNA before starting (see next page).

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Isolating mRNA, continued

Checking the Total RNA Quality

To check total RNA integrity, analyze 1 µg of your RNA by agarose gel electrophoresis. You should see the following on a denaturing agarose gel:

- 28S rRNA band (4.5 kb) and 18S rRNA band (1.9 kb) for mammalian species
- 28S band should be twice the intensity of the 18S band

Checking the mRNA Quality

To check quality, analyze 100 ng of your mRNA using a Bioanalyzer, or by performing agarose gel electrophoresis using a 1% E-Gel® EX Gel (see page 45 for ordering information). Your mRNA will appear as a smear, with the greatest intensity in the range of 1 to 3 kb. Some rRNA bands may still be faintly visible. If you do not detect a smear, or if the majority of the smear is significantly less than 1 kb, you will need to repeat the RNA isolation. Be sure to follow the recommendations listed on the previous page to prevent RNase contamination.

Checking the RNA Yield

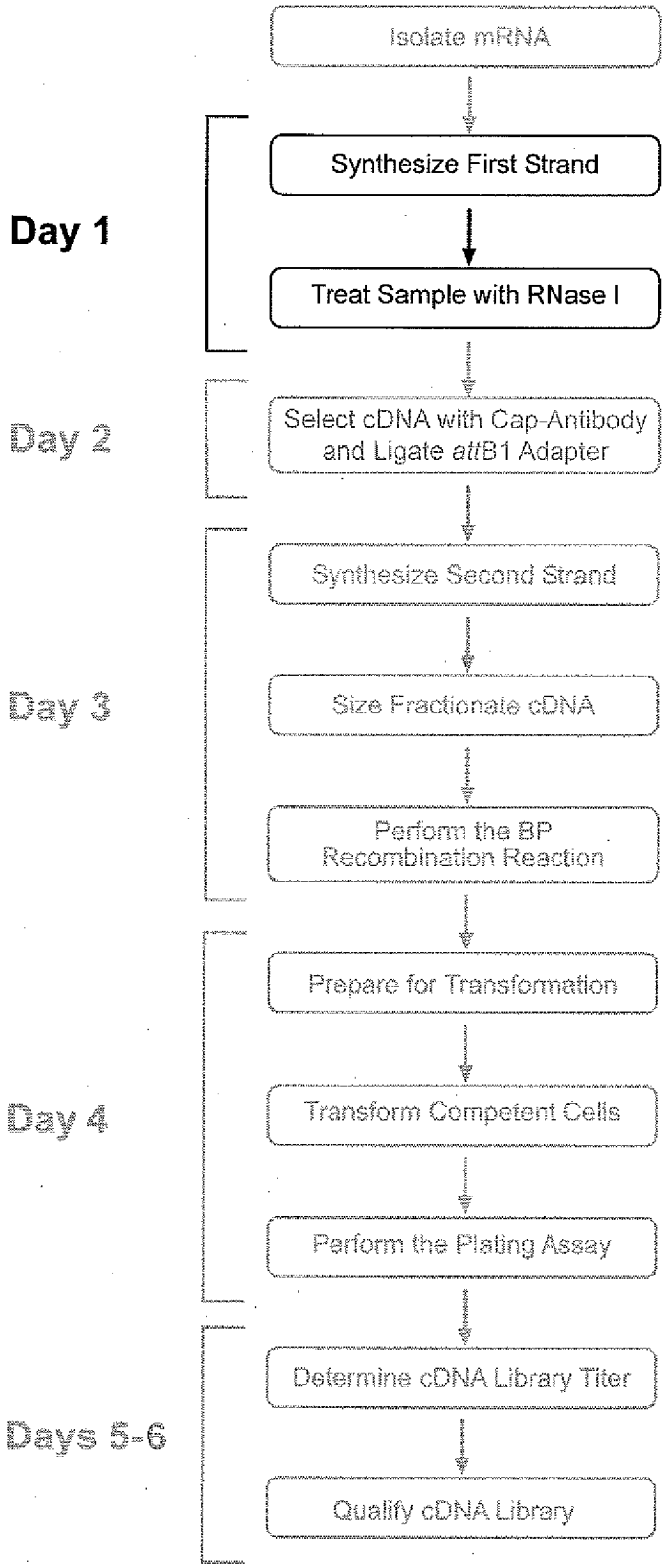
Use a NanoDrop or Bioanalyzer to check the yield of your RNA, or use the following general protocol to calculate the yield of total or mRNA using A_{260} absorbance:

1. Aliquot 2 µL of the RNA into a clean UV cuvette and add 198 µL of TE Buffer for a 1:100 dilution.
2. Blank a UV/visible spectrophotometer using TE Buffer, and then measure the sample at 260 nm.
3. The A_{260} reading should fall within the standard specification for the spectrophotometer (typically 0.01–1.0 OD). If it falls outside this range, adjust the dilution and rescan. If the A_{260} reading is too low, use a lower dilution; if it's too high, use a higher dilution.
4. Calculate the yield of RNA using the formula below:

   RNA yield (µg/µL) = A_{260} × 0.04 µg/µL RNA × Dilution factor

The dilution factor is 100 for the dilution described in this procedure. For example, if you diluted 2 µL of mRNA at 1:100, and the A_{260} is 0.5, then 0.5 × 0.04 µg/µL RNA × 100 = 2 µg/µL.

Day 1: Synthesizing First Strand cDNA and RNase I Digestion



Synthesizing the First Strand

Introduction

This section provides detailed guidelines for synthesizing the first strand of cDNA from your isolated mRNA. The reaction conditions for first strand synthesis catalyzed by SuperScript® III RT have been optimized for yield and size of the cDNAs.

Important

cDNA synthesis is a multi-step procedure requiring many specially prepared reagents which are crucial to the success of the process. Quality reagents necessary for converting your mRNA sample into double-stranded cDNA are provided with this kit. To obtain the best results, **do not substitute any of your own reagents for the reagents supplied with the kit.**

Starting mRNA

To successfully construct a cDNA library, it is crucial to start with high-quality mRNA. For guidelines on isolating mRNA, see page 7. The amount of mRNA needed to prepare a library depends on the efficiency of each step. Using this kit, anywhere from 5 µg to 10 µg of mRNA is used to construct cDNA libraries containing 10^6 to 10^7 primary clones in *E. coli*.

Guidelines

Consider the following points before performing the priming and first strand reactions:

- We recommend using no more than 10 µg of starting mRNA for the first strand synthesis reaction
- We recommend using a thermocycler rather than a water bath both for ease and for accurate temperatures and incubation times

If you are constructing multiple libraries, we recommend making a cocktail of reagents to add to each tube rather than adding reagents individually. This reduces the time required for the step and also reduces the chance of error.

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Synthesizing the First Strand, continued

Materials Required

Keep all reagents on ice until needed.

Supplied with kit:

- DEPC-treated water
- *att*B2-dT22VN Primer (1.5 µg/µL)
- 10 mM (each) dNTPs
- 5X First Strand Buffer
- 0.1 M DTT
- SuperScript® III RT (200 U/µL)

Supplied by user:

- High-quality mRNA (5–10 µg)
- Thermocycler (recommended) or heat block, heated to 70°C
- Ice bucket
- Thermocycler (recommended) or heat block, for stepwise incubations at 45°C, 50°C, and 55°C

Priming Reaction

Perform the priming reaction in a RNase-free 0.2 mL tube if using a thermocycler, or a 1.5 mL tube if using a heat block.

1. Add DEPC-treated water to your starting mRNA (5–10 µg) to a final volume of 25.5 µL.
2. Add the *att*B2-dT22VN Primer to your diluted mRNA, according to the table below:

Reagent	Volume
mRNA + DEPC-treated water	25.5 µL
*att*B2-dT22VN Primer (1.5 µg/µL)	2 µL

3. Mix the contents gently by pipetting.
4. Incubate the mixture at 70°C for 7 minutes and allow it to gradually cool to 45°C over 5–15 minutes, by ramping the temperature down (0.1°C/second) if using a PCR machine, or turning the heat off if using a heat block. During these incubation steps, perform Step 1 of the **First Strand Reaction**, next page.

continued on next page

Synthesizing the First Strand, continued

First Strand Reaction

1. Add the following reagents to a fresh RNase-free 1.5 mL tube:

Reagent	Volume
5X First Strand Buffer	10 µL
0.1 M DTT	5 µL
10 mM (each) dNTPs	2.5 µL
SuperScript® III RT (200 U/µL)	5 µL

   Mix the contents gently by pipetting, and place on ice until the priming reaction (Step 4, previous page) has cooled to 45°C.

2. Incubate the tube containing the first strand mixture (Step 1, above) at 45°C for 2 minutes.

3. Add the first strand mixture to the tube containing the priming reaction (previous page). **Be careful not to introduce bubbles into your sample.** The total volume should be 50 µL.

4. With the tube still in the thermocycler or heat block, mix the contents gently by pipetting. **Be careful to not introduce bubbles.**

5. Incubate the reaction tube in stepwise increments as follows:

45°C	20 minutes
50°C	30 minutes
55°C	30 minutes

6. Proceed to **Ethanol Precipitation**, page 13.

Synthesizing the First Strand, continued

Ethanol Precipitation

1. If the first strand reaction was carried out in a 0.2 mL tube, transfer the contents to a clean 1.5 mL tube. Add the following reagents to the tube in the following order:

Reagent	Volume
Glycogen (20 µg/µL)	1 µL
7.5 M NH_4OAc	25 µL
100% ethanol	187 µL

Note: You may stop at this point and store the tube at –20°C overnight if necessary.

2. Place the tube in dry ice or at –80°C for at least 1 hour. Centrifuge the sample at +4°C for 30 minutes at 16,000 × g.
3. Carefully remove the supernatant while trying not to disturb the cDNA pellet.
4. Add 150 µL of 70% ethanol. Centrifuge the sample at +4°C for 2 minutes at 16,000 × g, then carefully remove the supernatant.
5. Repeat Step 4 once. Remove as much of the remaining ethanol as possible.
6. Dry the cDNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.
7. Resuspend the pellet in 179 µL of DEPC-treated water by pipetting up and down 30–40 times.
8. Centrifuge for 2 seconds to collect the sample. Transfer the sample to a fresh tube and place on ice.
9. Proceed to **RNase I Treatment**, page 14.

RNase I Treatment

Introduction

This section provides guidelines for selecting full length cDNA.

Materials Required

You should have the following materials on hand before beginning. Keep all reagents on ice until needed.

Supplied with kit:

- RNase I (100 U/µL)
- 10X RNase I buffer
- Glycogen (20 µg/µL)
- TEN buffer
- 7.5 M NH_4OAc (ammonium acetate)

Supplied by user:

- Ice bucket
- Thermocycler (recommended) or water bath at 37°C
- Phenol:chloroform:isoamyl alcohol (25:24:1)
- 100% ethanol
- Dry ice or a –80°C freezer
- 70% ethanol

RNase I Treatment

1. Place the first strand reaction tube containing your cDNA on ice. Keep the tube on ice while adding the following reagents.

Reagent	Volume
Sample in DEPC-treated water	179 µL
10X RNase I buffer	20 µL
RNase I (100 U/µL)	1 µL

   The total volume should be 200 µL.

2. Mix the contents gently by pipetting.
3. Incubate at 37°C for 30 minutes.
4. Proceed to **Phenol/Chloroform Extraction**, below.

Phenol/Chloroform Extraction

1. Add 200 µL phenol:chloroform:isoamyl alcohol (25:24:1) and vortex or shake by hand thoroughly for approximately 20 seconds.
2. Centrifuge at room temperature for 5 minutes at 16,000 × g. Carefully remove the upper aqueous phase, and transfer the layer (~180 µL) to a fresh 1.5 mL tube. Be sure not to carry over any phenol during pipetting.
3. Proceed to **Ethanol Precipitation**, next page.

continued on next page

RNase I Treatment, continued

Ethanol Precipitation

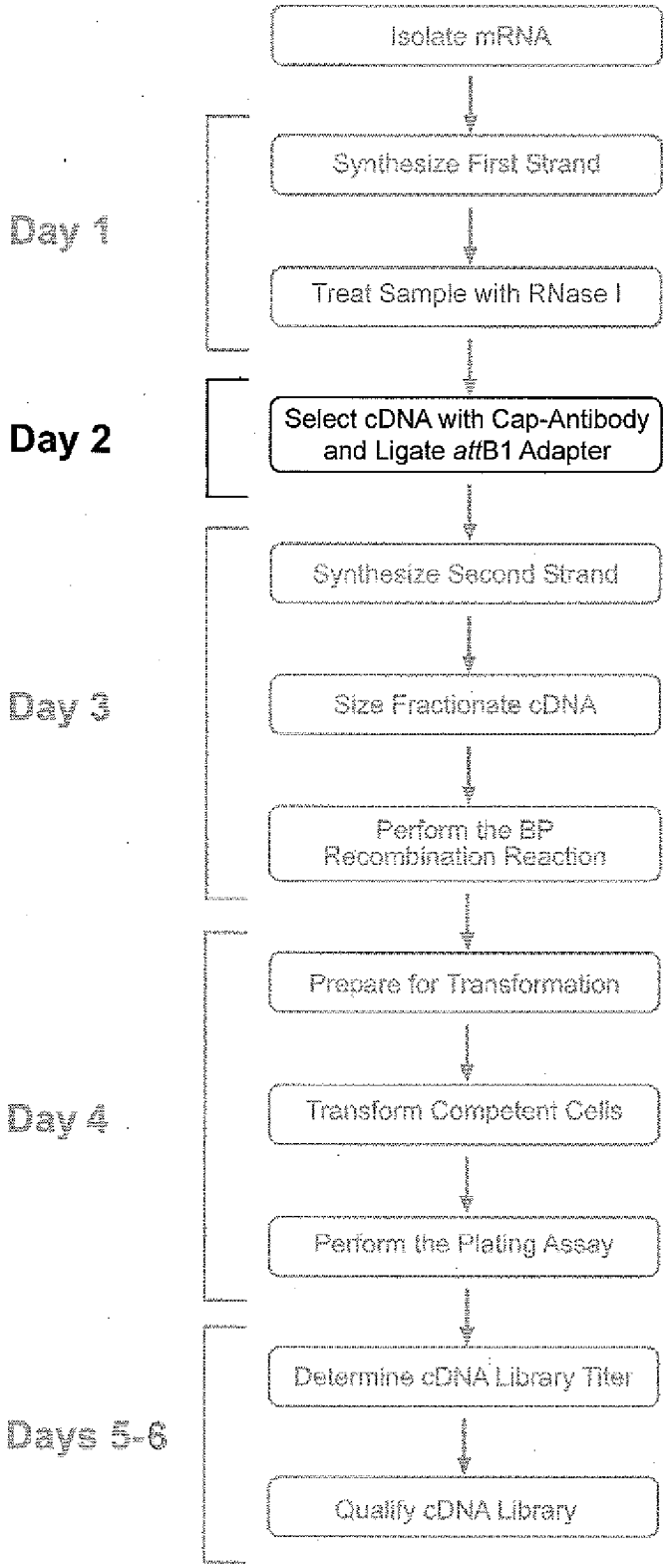
1. Add the following reagents to the aqueous phase (~180 µL), in the listed order:

Reagent	Volume	
Glycogen (20 µg/µL)	1 µL	
7.5 M NH_4OAc	90 µL	(*i.e.* 0.5 × volume of cDNA)
100% ethanol	675 µL	[*i.e.* 2.5 × (volume of cDNA+NH_4OAc)]

2. Place the tube at –20°C overnight to precipitate the cDNA.
Note: If you wish to continue with the protocol, place the tube in dry ice or at –80°C for at least 1 hour.

3. Proceed to **Ethanol Precipitation**, page 17.

Day 2: Cap Antibody Selection and Adapter Ligation



Cap Antibody Selection

Introduction

This section provides guidelines for selecting full length cDNA.

Materials Required

You should have the following materials on hand before beginning. Keep all reagents on ice until needed.

Supplied with kit:

- Cap-antibody beads
- TEN buffer
- Yeast tRNA
- TENG buffer
- 5 M NaOH (dilute to **50 mM** NaOH for use)
- 1 M Tris-HCl
- Spin Column
- Binding Buffer
- Wash Buffer (containing ethanol)
- DEPC-treated water

Supplied by user:

- Ice bucket
- Microcentrifuge
- 100% ethanol
- Magna-Sep™ Magnetic Particle Separator or other magnetic rack for 1.5 mL microfuge tubes

Ethanol Precipitation

1. Centrifuge the RNase I treated cDNA sample (page 15) at +4°C for 30 minutes at 16,000 × g.
2. Carefully remove the supernatant while trying not to disturb the cDNA pellet.
3. Add 150 µL of 70% ethanol. Centrifuge the sample at +4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant.
4. Repeat Step 3 once. Remove as much of the remaining ethanol as possible.
5. Dry the cDNA pellet in a SpeedVac® for 2 minute or at room temperature for 5–10 minutes.
6. Resuspend the cDNA pellet in 300 µL of TEN buffer by pipetting up and down 30–40 times.
7. Centrifuge for briefly to collect the sample, and place on ice.

continued on next page

Cap Antibody Selection, continued

Preparing 50 mM NaOH

1. Dilute 5 M NaOH for use in eluting cDNA from Cap-antibody beads as follows:

Reagent	Volume
5 M NaOH	5 µL
DEPC- water	495 µL

Do not use undiluted NaOH for the elution step.

Preparing Cap-antibody beads

1. Resuspend Cap-antibody beads and transfer 300 µL into a new tube.
2. Place the tube into the Magna-Sep™ Magnetic Particle Separator, and allow the beads to settle for 2 minutes.
3. Remove the supernatant, and resuspend the beads in another 300 µL of TEN buffer.
4. Place the tube into the Magnetic Particle Separator, and allow the beads to settle for 2 minutes.
5. Remove the supernatant, and resuspend the beads in 300 µL of TEN buffer with 20 µg (2 µL) of yeast tRNA. Incubate for 10 minutes at room temperature.
6. Proceed to **Cap Antibody Selection**, below.

Cap Antibody Selection

1. Mix the Cap-antibody beads and add them to the cDNA sample (page 17, Step 7).
2. Incubate for 1 hour at room temperature. Mix the beads gently with a pipette tip after 30 minutes to keep them suspended.
3. Place the tube into the Magna-Sep™ Magnetic Particle Separator, and allow the beads to settle for 1 minute. Remove and discard the supernatant.
4. Resuspend the beads in 300 µL of TEN buffer.
5. Place the tube into the Magnetic Particle Separator, and allow the beads to settle for 1 minute. Remove and discard the supernatant.
6. Repeat Steps 4–5 two more times.
7. Resuspend the beads with 300 µL of TENG buffer, transfer the beads to a fresh tube, and incubate for 5–10 minutes.
8. Proceed to **Elution from Cap Antibody Beads**, next page.

continued on next page

Cap Antibody Selection, continued

Elution from Cap Antibody Beads

1. Before eluting your sample, label a new 1.5 mL collection tube for your full-length cDNA. Add 100 µL 1 M Tris-HCl (pH 7.0) to the collection tube.
2. Remove and discard the supernatant from the Cap-antibody beads.
3. Add 100 µL of **50 mM** NaOH (see **Preparing 50 mM NaOH**, page 18) to the beads. Mix and incubate for 10 minutes at 37°C to elute cDNA from the beads.
4. Place the tube into the Magna-Sep™ Magnetic Particle Separator, and allow the beads to settle for 1 minute.
5. Transfer the supernatant into the labeled collection tube containing 100 µL 1 M Tris-HCl (pH 7.0) to neutralize the NaOH.
6. Repeat Steps 3–5 two more times. The supernatants from each additional elution is collected in the same tube containing the first elution. The final volume after the third elution should be 400 µL.
7. Proceed to **cDNA Purification**, below.

Preparing Wash Buffer

For new kits, prepare the Wash Buffer with 100% ethanol as follows:

1. Add 10 mL of 100% ethanol to the Wash Buffer.
2. Check the box on the Wash Buffer label to indicate that ethanol was added.

cDNA Purification

1. Add 400 µL Binding Buffer to the collected eluate.
2. Add 400 µL 100% ethanol to the collected eluate.
3. Transfer 600 µL of the sample into the Spin Column in a 2 mL waste collection tube.
4. Centrifuge for 1 minute at 8,100 × g (~10,000 rpm). Discard the flow-through.
5. Transfer the remaining 600 µL of the sample into the same Spin Column and centrifuge for 1 minute at 8,100 × g. Discard the flow-through.
6. Add 400 µL Wash Buffer. Centrifuge for 1 minute at 8,100 × g.
7. Add 400 µL Wash Buffer. Centrifuge for 1 minute at 16,000 × g (14,000 rpm) to remove all of the Wash Buffer.
8. Elute the cDNA by placing the Spin Column into a 1.5 mL collection tube, and adding 12 µL DEPC-treated water in the center of the column.
9. Incubate for 1 minute. Centrifuge for 1 minute at 16,000 × g.
10. Perform a second elution with 12 µL DEPC-treated water. Incubate for 1 minute. Centrifuge for 1 minute at 16,000 × g.
11. Proceed to **Ligating the *att*B1 Adapter**, next page.

Ligating the *att*B1 Adapter

Introduction

Follow the guidelines in this section to ligate the adapters in the attB1 Adapter Mix to the 5′ end of your first strand cDNA.

Materials Required

Keep all reagents on ice until needed.

Supplied with kit:

- 5X Adapter Buffer
- *att*B1 Adapter Mix (0.5 µg/µL)
- 0.1 M DTT
- T4 DNA Ligase (1 U/µL)

Supplied by user:

- Ice bucket
- 100% ethanol
- Thermocycler (recommended) or water bath at 16°C

Ligation Protocol

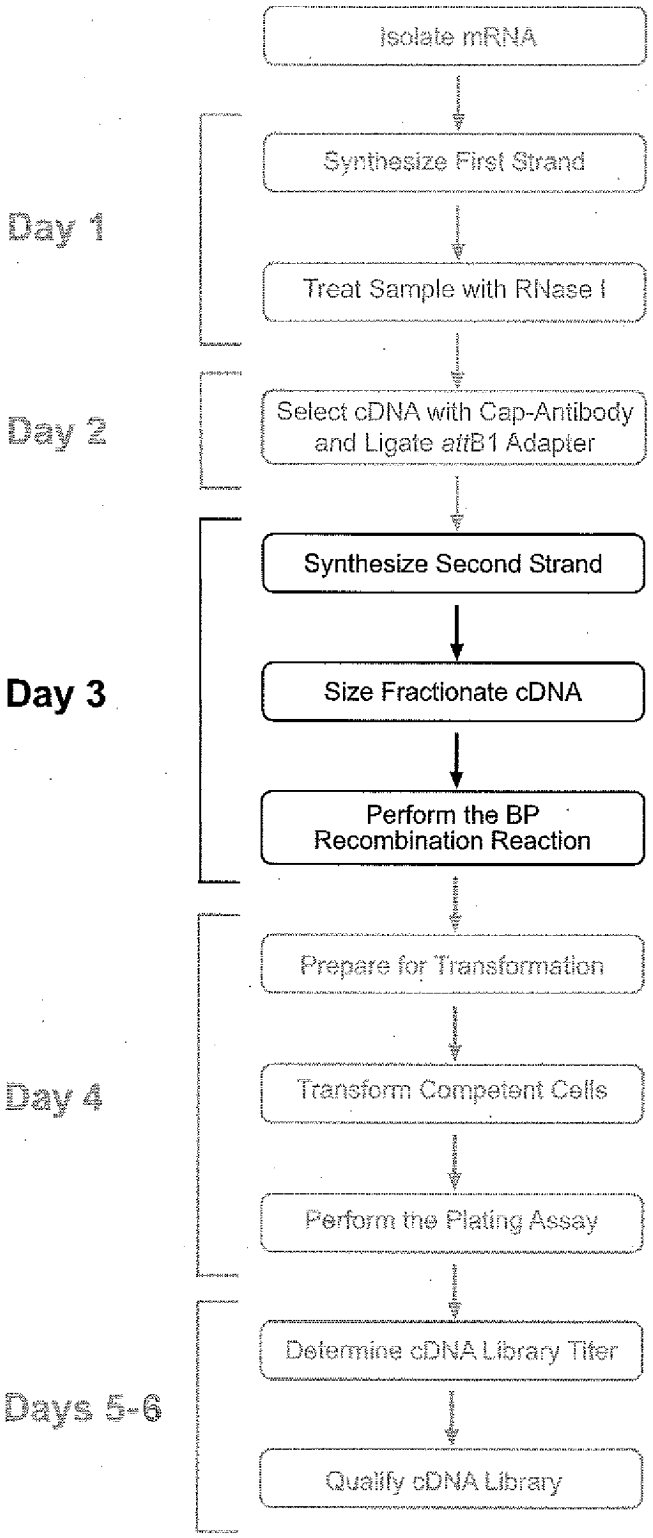
1. Take your eluted first strand cDNA (~22 µL) from Step 10, page 19 and add the following reagents:

Reagent	Volume
5X Adapter Buffer	10 µL
*att*B1 Adapter Mix (0.5 µg/µL)	5 µL
0.1 M DTT	8 µL
T4 DNA Ligase (1 U/µL)	5 µL

2. Mix the contents gently by pipetting. Incubate at 16°C for 16–24 hours.
3. Proceed to **cDNA Purification**, page 22.

continued on next page

Day 3: Second Strand cDNA Synthesis, Size Fractionation by Column Chromatography and Performing the BP Recombination Reaction



Synthesizing the Second Strand

Introduction

This section provides guidelines for primer extension-based second-strand synthesis of the adapter ligated first strand cDNA using the 5′ Primer provided in the kit.

Materials Required

You should have the following materials on hand before beginning. Keep all reagents on ice until needed.

Supplied with kit:

- Spin Column
- Binding Buffer
- Wash Buffer (containing ethanol)
- DEPC-treated water
- 5′ Primer
- 10 mM (each) dNTPs

Supplied by user:

- Ice bucket
- Microcentrifuge
- Thermocycler
- 10X High Fidelity PCR Buffer
- 50 mM $MgSO_4$
- Platinum® *Taq* DNA Polymerase High Fidelity (or other high fidelity polymerase capable of synthesizing long length cDNA)

cDNA Purification

1. Add 50 µL Binding Buffer to the ligation mixture.
2. Add 50 µL 100% ethanol to the ligation mixture.
3. Transfer the ligation mixture into a Spin Column in a 2 mL waste collection tube.
4. Centrifuge for 1 minute at 8,100 × g (10,000 rpm). Discard the flow-through.
5. Add 200 µL Wash Buffer. Centrifuge for 1 minute at 8,100 × g.
6. Add 200 µL Wash Buffer. Centrifuge for 1 minute at 16,000 × g (14,000 rpm).
7. Elute the cDNA by placing the Spin Column into a 1.5 mL collection tube, and adding 40 µL DEPC-treated water in the center of the column.
8. Incubate for 1 minute. Centrifuge for 1 minute at 16,000 × g.
9. Perform a second elution with 40 µL DEPC-treated water. Incubate for 1 minute. Centrifuge for 1 minute at 16,000 × g.
10. Proceed to **Synthesizing the Second Strand,** next page.

Synthesizing the Second Strand, continued

Second Strand Reaction

1. Place the first strand reaction tube containing your cDNA on ice. Keep the tube on ice while adding the following reagents.

Reagent	Volume
cDNA with 5′ *att*B1 Adapter	79 µL
10X High Fidelity PCR Buffer	10 µL
10 mM (each) dNTPs	4 µL
50 mM $MgSO_4$	5 µL
5′ Primer (100 ng/µL)	1 µL
Platinum® *Taq* DNA Polymerase High Fidelity	1 µL

   The total volume should be 100 µL reactions.

2. Mix the contents gently by pipetting.

3. Incubate the reaction tube as follows:

68°C	20 minutes
72°C	20 minutes
4°C	hold

   Note: Do not perform a denaturation step for this reaction, as it causes dissociation of the double-stranded adapter sequences.

4. Proceed to **Size Fractionating cDNA by Column Chromatography**, next page.

Size Fractionating cDNA by Column Chromatography

Column Chromatography

Column chromatography is commonly used to size fractionate cDNA. This method makes the cloning of larger inserts more probable, and generates a cDNA library with an average cDNA insert size of approximately 1.0 kb (when starting with high-quality mRNA).

Columns are provided with the kit. Follow instructions closely using the columns supplied with the kit to produce the highest quality library possible.

How the Columns Work

Each column provided with the kit contains 1 mL of Sephacryl® S-500 HR resin. This porous resin traps residual adapters, primers, and/or small truncated cDNAs (<500 bp) and prevents them from contaminating the library. Larger molecules bypass the resin and elute quickly while smaller molecules are retained within the resin and elute more slowly. Thus, earlier eluted fractions contain larger cDNA fragments than later fractions.

Materials Required

Supplied with kit:

- cDNA Size Fractionation Columns
- Glycogen (20 µg/µL)
- TEN buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 25 mM NaCl)
- 7.5 M NH_4OAc (ammonium acetate)

Supplied by user:

- cDNA sample
- 100% ethanol
- Dry ice or –80°C freezer
- 70% ethanol
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)

continued on next page

Size Fractionating cDNA by Column Chromatography, continued

Important

If you are constructing more than one cDNA library, only add one cDNA adapter ligation reaction per column.

Setting Up the Column

Keep the following points in mind when setting up a fractionation column:

- Anchor the column securely in a support stand
- Place a rack containing 1.5 mL tubes below the column
- The outlet of the column should be 1 to 2 cm above the 1.5 mL tubes
- You will need to be able to freely move the rack under the column

Washing the Column

cDNA size fractionation columns are packed in 20% ethanol which must be completely removed before adding your cDNA sample. Follow the steps below to remove the ethanol from the columns. The washing steps take approximately 1 hour.

1. With the column attached to a support stand, remove the **top cap first** followed by the bottom cap. Allow the ethanol to drain completely by gravity.
2. Once the column stops dripping, pipette 0.8 mL of TEN buffer into the column and let it drain completely. Refer to the important note below for column specifications.
3. Repeat the wash step three more times for a total of four washes (3.2 mL) of TEN buffer. Let the column drain until dry. Proceed to **Collecting Fractions**, next page.

Important

If the flow rate is noticeably slower than 30–40 seconds per drop, or the drop size from the column is not approximately 25 to 35 µL, do not use the column. The integrity and resolution of the cDNA may be compromised if the column does not meet these specifications.

continued on next page

Size Fractionating cDNA by Column Chromatography, continued

- When collecting fractions, wear gloves that have been rinsed with ethanol to reduce static
- Make sure all of the effluent has drained from the column before adding each new aliquot of TEN buffer

Collecting Fractions

1. Label 3 sterile 1.5 mL tubes from 1 to 3. Place them in a rack 1 to 2 cm from the bottom of the column with tube 1 under the outlet of the column.
2. Add 50 µL of TEN buffer to the 100 µL cDNA adapter ligation reaction from Step 4, page 23. Mix gently by pipetting.
3. Add the entire sample to the column and let it drain into the resin bed. Collect the effluent into Tube 1.
4. Add another 100 µL of TEN buffer to the column and let it drain into the resin bed. Collect the effluent in Tube 1.
5. Move Tube 2 under the column outlet and add 240 µL of TEN buffer to the column. Collect the effluent into Tube 2. Let the column drain completely.
   Note: To enrich for larger cDNA, use 160 µL of TEN buffer instead of 240 µL for elution into Tube 2.
6. Move Tube 3 under the column outlet and add 80 µL of TEN buffer to the column. Collect the effluent into Tube 3. Let the column drain completely.
7. Tube 2 and 3 contain double strand cDNA molecules. The largest molecules are eluted from the column first, Tube 2 contains more large-size cDNA than Tube 3. Usually, cDNA eluted in Tube 2 are enough to generate a representative cDNA library that contains 10^6 primary clones, but occasionally, more cDNA may elute in the subsequent fraction. Keep Tube 3 for possible later use, depending upon the result of your library from Tube 2 and amount of starting mRNA.
8. *Optional*: Determine your cDNA quantity using a Bioanalyzer or NanoDrop.
9. Take Tube 2 and/or Tube 3 (see below) and proceed to **Ethanol Precipitation**, next page.

Important

Save the sample in Tube 3. **Do not combine** Tube 3 with Tube 2, as Tube 3 may be potentially contaminated with adapters and primers which may result in background colonies upon plating (i.e., vectors with only *att*B1 and *att*B2, or polyA sequence inserts).

Generate an independent cDNA library from the contents of Tube 3 when:

- The starting mRNA is much less than 5 µg
- The library does not contain the requisite number of primary clones
- Smaller cDNA inserts are needed (this protocol does not work for cloning of small RNA or miRNA)

continued on next page

Size Fractionating cDNA by Column Chromatography, continued

Ethanol Precipitation

1. To the tube of pooled cDNA, add reagents in the following order:

Reagent	Amount	
Glycogen (20 µg/µL)	1 µL	
7.5 M NH_4OAc	0.5 volume	(*i.e.* 0.5 × volume of cDNA)
100% ethanol	2.5 volumes	[*i.e.* 2.5 × (volume of cDNA +NH_4OAc)]

Note: You may stop at this point and store the tube at –20°C overnight if necessary.

2. Place the tube in dry ice or at –80°C for at least 1 hour. Centrifuge the sample at +4°C for 30 minutes at 16,000 × g.
3. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150 µL of 70% ethanol.
4. Centrifuge the sample at +4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.
5. Dry the cDNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.
6. Resuspend the cDNA pellet in 7 µL of TE buffer or DEPC water by pipetting up and down 30–40 times.

   Note: If you perform analysis of your cDNA yield, you may wish to resuspend the cDNA pellet in a larger volume of TE buffer so that less cDNA is used up for analysis. Take the volumes of reagents required to perform the BP reaction into account when determining the volume for resuspending your cDNA,

Estimating the cDNA Yield

If desired, determine the yield of the cDNA after column purification and ethanol precipitation. Note that 1 µL of your cDNA will be used in this procedure, reducing the amount of cDNA that you have to perform the BP recombination reaction.

Determine yield by analyzing 1 µL of your cDNA (from Step 6, above) using a Bioanalyzer, NanoDrop, or by performing agarose gel electrophoresis using a 1% E-Gel® EX Gel (see page 45 for ordering information).

What You Should See

A typical final cDNA yield is approximately 50–500 ng, starting from 5–10 µg of starting mRNA. Using 50–500 ng of cDNA in the BP reaction should produce a library containing 1–10 million clones.

If your cDNA yield is less than 50 ng, you may generate a second cDNA library using the contents from Tube 3 (see previous page, Step 6).

continued on next page

Performing the BP Recombination Reaction

Introduction

General guidelines are provided below to perform a BP recombination reaction between your *att*B-flanked cDNA and pDONR™ 222 to generate a Gateway® entry library. For a map and a description of the features of pDONR™ 222, see pages 52-53.

Propagating pDONR™ 222

If you wish to propagate and maintain pDONR™ 222, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (Cat. no. A10460) from Invitrogen. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 50 µg/mL kanamycin and 30 µg/mL chloramphenicol.

Note: DO NOT use general *E. coli* cloning strains including TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to CcdB effects. **DO NOT** use the ElectroMAX™ DH10B™ competent cells provided with this kit.

Materials Required

Keep all reagents on ice until needed.

Supplied with kit:

- pDONR™ 222 (150 ng/µL)
- BP Clonase™ II enzyme mix (keep at –20°C until immediately before use)

Supplied by user:

- *att*B-flanked cDNA (75–100 ng)
- TE buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)
- 25°C incubator

continued on next page

Performing the BP Recombination Reaction, continued

BP Recombination Reaction

Perform the BP recombination reaction using the *att*B-flanked cDNA insert with a pDONR™ 222 vector in a total of 11–12 µL.

If your cDNA yield is less than 100 ng use 150 ng (1 µL) pDONR™ 222 vector. If you have more than 100 ng cDNA use 300 ng (2 µL) pDONR™ 222 vector. If your cDNA yield is less than 50 ng, then perform a separate BP reaction with Tube 3.

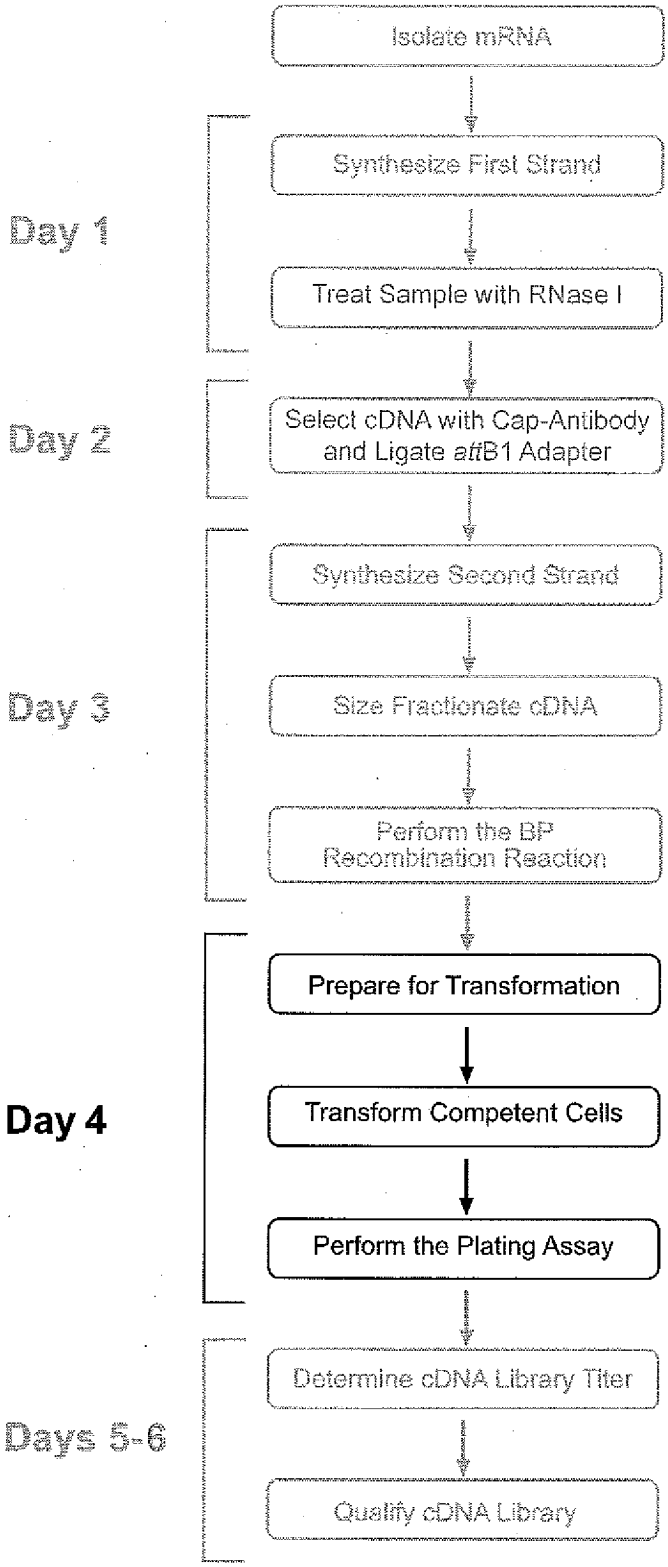
1. Add the following components to a sterile 1.5 mL microcentrifuge tube at room temperature and mix.

Reagent	Volume
*att*B-flanked cDNA	7 µL
pDONR™ 222 (150 ng/µL)	1–2 µL
TE buffer, pH 8.0 (or DEPC water)	to 7 µL

2. Remove the BP Clonase™ II enzyme mix from –20°C and thaw on ice (~2 minutes).
3. Vortex the BP Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. Add 3 µL of BP Clonase™ II enzyme mix to each sample. Mix the contents gently by pipetting. The total volume in each tube should now be 11–12 µL.

   Reminder: Return BP Clonase™ II enzyme mix to –20°C immediately after use.
5. Incubate reactions at 25°C for 16–20 hours. Proceed to **Day 4: Transforming Competent Cells**, page 31.

Day 4: Transforming Competent Cells



Preparing for Transformation

Introduction

Once you have performed the BP recombination reaction, you will inactivate the reaction with proteinase K, ethanol precipitate the cDNA, and transform it into competent *E. coli*. The ElectroMAX™ DH10B™ T1 Phage Resistant Cells provided with the kit have a high transformation efficiency (>1 × 10^{10} cfu/µg DNA) making them ideal for generating cDNA libraries. Follow the guidelines below to prepare for the transformation procedure.

Transformation Control

If you are performing library construction for the first time, it is recommended to perform a control reaction. A tube of pUC19 plasmid is included in the kit to check the transformation efficiency of ElectroMAX™ DH10B™ T1 Phage Resistant Cells. Transform 10 pg of pUC19 using the protocol on page 34.

Materials Required

Supplied with kit:

- Proteinase K (2 µg/µL)
- Glycogen (20 µg/µL)
- pUC19 positive control (10 pg/µL)
- 7.5 M NH_4OAc (ammonium acetate)

Supplied by user:

- BP recombination reactions (from Step 5, page 29)
- Water bath, heated to 37°C
- Thermocycler or water bath, heated to 75°C
- Sterile water
- 100% ethanol
- Dry ice or a –80°C freezer
- 70% ethanol
- 15 mL snap-cap tubes (*e.g.* Falcon™ tubes)
- Ice bucket

continued on next page

Preparing for Transformation, continued

Stopping the BP Recombination Reaction

1. Add 2 µL of proteinase K to each BP reaction from Step 5, page 29, to inactivate the BP Clonase™ II enzyme mix.
2. Incubate the reactions at 37°C for 15 minutes then at 75°C for 10 minutes.

Ethanol Precipitation

1. Add sterile water to the BP reaction mix to bring it to a final volume of 100 µL.
2. Add reagents in the following order to each tube:

Reagent	Amount
Glycogen (20 µg/)	1 µL
7.5 M NH_4OAc	50 µL
100% ethanol	375 µL

Note: You may stop at this point and store the tube at –20°C overnight if necessary.

3. Place the tube in dry ice or at –80°C for 1 hour. Centrifuge the sample at +4°C for 25 minutes at 16,000 × g.
4. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150 µL of 70% ethanol.
5. Centrifuge the sample at +4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.
6. Dry the cDNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.
7. Resuspend the cDNA pellet in 10 µL of TE buffer or DEPC water by pipetting up and down 30–40 times.

Aliquoting Samples

1. Label four 1.5 mL tubes for each cDNA library sample. For example, if you are constructing multiple libraries, label tubes for library A: A1, A2, A3, *etc.*
2. Label one 1.5 mL tubes for the pUC19 transformation control.
3. For each 1.5 mL tube from Steps 1 and 2, label a duplicate 15 mL snap-cap tube (*e.g.* Falcon™ tube).
4. Aliquot cDNA library samples and controls into the appropriate tubes according to the table below. Place tubes on ice.

	cDNA Library	pUC19 Control
Number of 1.5 mL Tubes	4	1
Aliquot in Each Tube	2.5 µL	1.0 µL

5. Proceed to **Transforming ElectroMAX™ DH10B™ T1 Phage Resistant Cells,** next page.

continued on next page

Transforming ElectroMAX™ DH10B™ T1 Phage Resistant Cells

Important

Each box of ElectroMAX™ DH10B™ T1 Phage Resistant Cells consists of 5 tubes containing 100 µL of competent cells each. Each tube contains enough competent cells to perform 2 transformations using 50 µL of cells per transformation.

Use 2 tubes of competent cells for each library, and one tube for the control. Thaw cells by loosening the cap and placing the tube on ice for 5–10 minutes. Do not let the cells thaw for longer than 20 minutes, or a significant reduction of efficiency will occur.

Once you have thawed a tube of competent cells, discard any unused cells. **Do not** re-freeze cells as repeated freezing/thawing of cells may result in loss of transformation efficiency.

Materials Required

Supplied with kit:

- ElectroMAX™ DH10B™ T1 Phage Resistant Cells (thaw on ice before use)
- S.O.C. medium (Invitrogen, Cat. no. 15544-034)

Supplied by user:

- Ice bucket
- 0.1 cm cuvettes (on ice)
- Electroporator
- 37°C shaking incubator
- 15 mL snap-cap tubes (*e.g.* Falcon™ tubes)
- Freezing media (60% S.O.C. medium:40% glycerol, see below for recipe)

Freezing Media

60% S.O.C. medium:40% glycerol

1. Combine 60 mL of S.O.C. medium and 40 mL of glycerol and stir until solution is homogeneous.
2. Autoclave for 30 minutes on liquid cycle.
3. Store at room temperature for up to 1 month.

Electroporator Settings

If you are using the BioRad Gene Pulser® II or BTX® ECM® 630, we recommend the following settings:

Voltage	2.2 kV*
Resistance	200 Ω
Capacity	25 µF

*If the sample arcs at this voltage setting, recover the sample from the cap by tapping the cuvette, and redo the electroporation on the same cuvette at 2.0 kV.

If you are using another electroporator, you will need to optimize your settings using the pUC19 control DNA provided with the kit. The transformation efficiency of the ElectroMAX™ DH10B™ T1 Phage Resistant Cells should be at least 1×10^{10} cfu/µg of pUC19 control DNA.

continued on next page

Transforming ElectroMAX™ DH10B™ T1 Phage Resistant Cells, continued

Electroporation

We recommend that you electroporate your control first followed by your cDNA samples (four samples per library). This will allow you to troubleshoot any arcing problems before you electroporate your cDNA samples (see recommendation below).

1. Add 50 µL of thawed ElectroMAX™ DH10B™ competent cells to each tube containing a DNA aliquot. **Mix gently by pipetting up and down two times. Be careful to not introduce bubbles into your sample.**
2. Transfer the entire contents of each tube from Step 1, above, to a separate pre-chilled 0.1 cm cuvette. Distribute the contents evenly by gently tapping each side of the cuvette. **Be careful to not introduce bubbles into your sample**. Store the cuvette on ice.
3. Once the competent cells have been added to all four DNA aliquots, electroporate the samples simultaneously using your optimized setting (see **Electroporator Settings**, previous page). If the control arcs, repeat the pUC19 control electroporation using the same settings. If your sample arcs, you can try to recover the sample from the cap by tapping the cuvette, and redo the electroporation on the same cuvette at 2.0 kV.
4. Add 1 mL of S.O.C. medium to the cuvette containing electroporated cells. Using a pipette, transfer the entire solution to a labeled 15 mL snap-cap tube.
5. Shake electroporated cells for at least 1 hour at 37°C at 225–250 rpm to allow expression of the kanamycin resistance marker.
6. After the one hour incubation at 37°C, pool all cells representing one library into a 15 mL snap-cap tube.
7. Determine the volume for all cDNA libraries and add an equal volume of sterile freezing media (60% S.O.C. medium:40% glycerol). Mix by vortexing. Keep on ice. **This is the final cDNA library.**
8. Remove a 100 µL sample from each library and pUC19 control and place in 1.5 mL tubes for titer determination. Keep on ice.
9. **Store cDNA libraries at –80°C.** You may divide your library into multiple tubes to reduce the number of freeze/thaw cycles.
10. Proceed to **Performing the Plating Assay**, page 35.

If you experience arcing during transformation, try one of the following:

- Make sure the contents are distributed evenly in the cuvette and there are no bubbles.
- Reduce the voltage normally used to charge your electroporator by 10%.
- Make sure to ethanol precipitate the BP reaction prior to electroporation to reduce the salt concentration.
- Dilute the 1.5 µL aliquots with water and divide the sample in two. Electroporate extra samples of competent cells. Make sure that you have enough ElectroMAX™ DH10B™ Cells to perform this troubleshooting step (see page 45 for ordering information).

Performing the Plating Assay

Materials Required

Supplied by user:

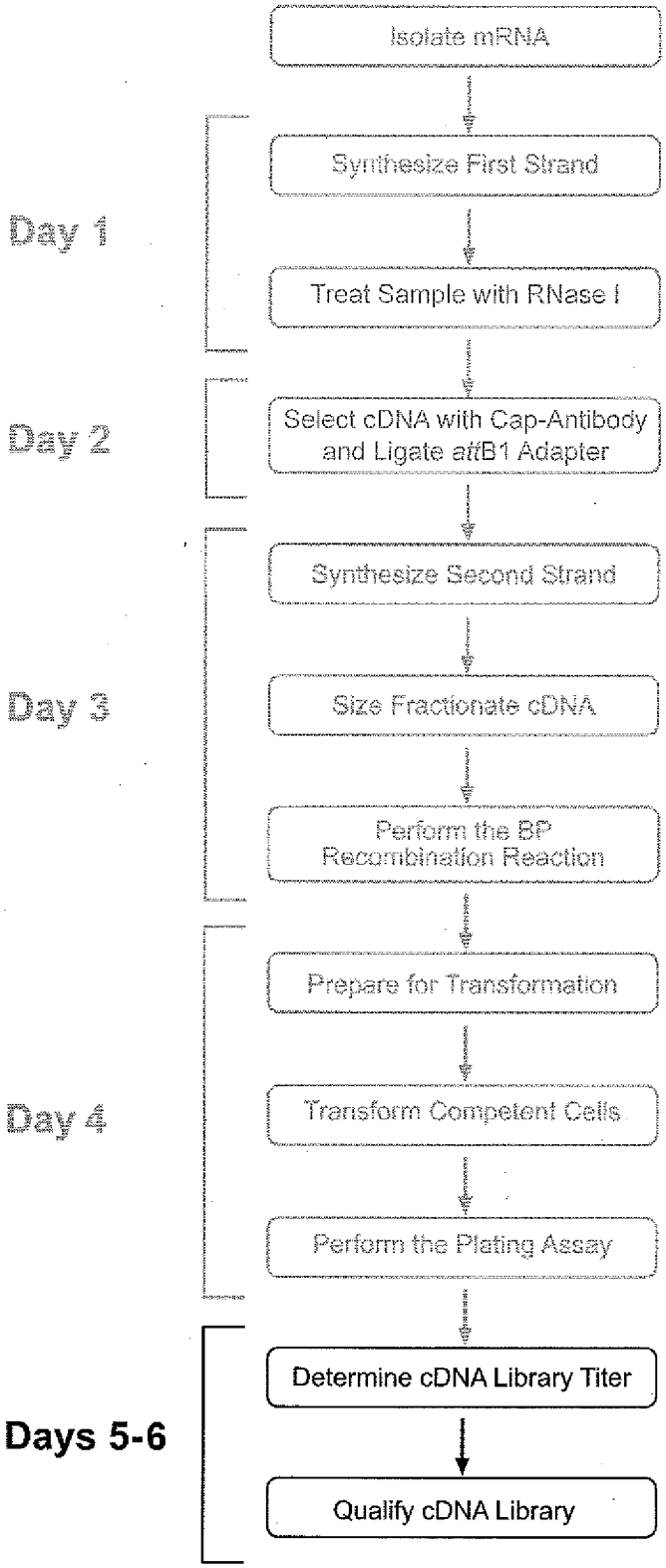
- cDNA library and pUC19 control aliquots
- S.O.C. medium (Invitrogen, Cat. no. 15544-034)
- LB plates containing 50 µg/mL kanamycin (six for each cDNA library and BP reaction controls, warm at 37°C for 30 minutes)
- LB plates containing 100 µg/mL ampicillin (two for pUC19 control, warm at 37°C for 30 minutes)

Plating Assay

1. Serially dilute your sample aliquots with S.O.C. medium according to the table below. For each 1:10 serial dilution, add 100 µL of the sample to 900 µL of S.O.C. medium.
2. You will be plating your serial dilutions in duplicate. You will need six prewarmed LB plates containing 50 µg/mL kanamycin for each cDNA library. You will need two prewarmed LB plates containing 100 µg/mL ampicillin for the pUC19 transformation control.
3. Plate 100 µL of each dilution onto prewarmed LB plates containing the appropriate antibiotic.
4. Incubate plates overnight at 37°C.
5. Proceed to **Days 4-5: Analyzing the cDNA Library**, next page.

	cDNA Library	pUC19 Control
Dilutions	10^{-2}	10^{-2}
	10^{-3}	--
	10^{-4}	--
Amount to Plate of Each Dilution	2 × 100 µL	2 × 100 µL
Total Number of LB + Kan Plates	6	--
Total Number of LB + Amp Plates	--	2

Days 5-6: Analyzing the cDNA Library



Determining the cDNA Library Titer

Calculations

1. Using the results from the plating assay, page 35, and the equation below, calculate the titer for each plate.

$$\textbf{cfu/mL} = \frac{\textit{colonies on plate} \times \text{dilution factor}}{\text{volume plated (mL)}}$$

Example:

If 15 colonies are counted on a plate containing 0.1 mL of a 10^{-3} dilution, the equation would be written as follows:

$$\textbf{cfu/mL} = \frac{15 \times 10^{3}}{0.1} = \mathbf{1.5 \times 10^{5}\ cfu/mL}$$

2. Use the titer for each plate to calculate the average titer for the entire cDNA library.
3. Use the average titer and the equation below to determine the total number of colony-forming units.

   Total CFU (cfu) = average titer (cfu/mL) × total volume of cDNA library (mL)

   Note: If you completed four electroporations for your cDNA library, the total volume will be 8 mL.

Expected Total CFUs

In general, a well represented library should contain 1×10^{6} to 1×10^{7} primary clones. If the number of primary clones is considerably lower for your cDNA library, see **Troubleshooting**, page 49.

What You Should See

See the table below for expected titers and expected total colony-forming units for the control reaction.

Control	Expected Titer	Expected Volume	Expected Total CFUs
pUC19 control	≥1×10^{10} cfu/µg DNA	--	--

Qualifying the cDNA Library

Introduction

It is important to qualify the cDNA library to determine the success of your cDNA library construction. Determining the average insert size and percentage of recombinants will give you an idea of the representation of your cDNA library.

General Molecular Biology Techniques

For help with restriction enzyme analysis, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Materials Required

Supplied by user:

- Restriction enzyme BsrG I, or Not I and EcoR I , and appropriate buffers
- 1 Kb Plus DNA Ladder, recommended (Invitrogen, Cat. no. 12302-011). Other DNA ladders are suitable.
- Electrophoresis apparatus and reagents

Analyzing Transformants by Restriction Enzyme Digestion

Average insert size and percentage of recombinants in the library can be determined with BsrG I digestion, or Not I /EcoR I double digestion. BsrG I sites generally occur at a low frequency making it an ideal restriction enzyme to use for insert size analysis.

BsrG I cuts within the *attL* sites of your entry clone to give you the size of your insert (see page 41 for a diagram of the recombination region)

Restriction Digest

We recommend that you analyze a minimum of 24 positive clones to accurately determine average insert size and the percentage of recombinants.

1. Pick 24 colonies from the plating assay and culture overnight in 1–2 mL LB containing 50 µg/mL of kanamycin.
2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink™ Quick Plasmid MiniPrep Kit (Cat. no. K2100-10) or the PureLink™ 96 Plasmid Purification System (Cat. no. 12263-018) if you will be analyzing multiple libraries at a time.
3. Digest 300–500 ng of plasmid DNA with Not I/Eco RI or *Bsr*G I following the manufacturer's instructions.
4. Electrophorese samples using a 1% agarose gel. Include a DNA ladder to help estimate the size of your inserts.

continued on next page

Qualifying the cDNA Library, continued

Expected Digestion Patterns

- The pDONR™ 222 control will show a digestion pattern of 3 bands of the following lengths:

  2.5 kb
  1.4 kb
  790 bp

- Each cDNA entry clone should have a vector backbone band of 2.5 kb and additional insert bands
- Make sure to digest enough plasmid DNA to be able to visualize smaller insert bands (<300 bp)
- Make sure to run the gel long enough to distinguish bands representing insert sizes of approximately 2.5 kb from the 2.5 kb vector backbone band
- If only a single 2.5 kb band is visible, the insert may be too small to be visible (e.g. a primer), or the insert may also be 2.5 kb. PCR can be used to verify the size of the insert in these instances.

Determining Average Insert Size and % Recombinants

1. Identify clones containing inserts using the guidelines outlined above.
2. For clones containing inserts, use the DNA ladder to estimate band sizes. If there are multiple bands for a single cDNA entry clone, add all band sizes to calculate the insert size. Do not include the 2.5 kb vector backbone band in your calculations. Refer to page 51 for sample results.
3. Add the insert sizes for all clones. Divide this number by the number of clones containing inserts to calculate the average insert size for your cDNA library.
4. Divide the number of clones containing inserts by the number of clones analyzed to determine the percent recombinants.

What You Should See

For **standard cDNA libraries**, you should see an average insert size of ≥1 kb and at least 87% recombinants. For libraries generated from the contents of the column fraction in Tube 3 (page 26), the size will be smaller.

If the average insert size or percent recombinants of your library clones is significantly lower, the cDNA going into the BP recombination reaction is either of poor quality or is insufficient in quantity. For guidelines on isolating quality mRNA, see page 7. To troubleshoot any of the cDNA synthesis steps, see **Troubleshooting**, page 49.

The Next Step

To sequence entry clones, proceed to **Sequencing Entry Clones**, next page.

You may screen your cDNA library to identify a specific entry clone and use this entry clone in an LR recombination reaction with a destination vector to generate an expression clone. Refer to the Gateway® Technology manual to perform an LR recombination reaction using a single entry clone.

Alternatively, you may transfer your cDNA library into a destination vector to generate an expression library for functional analysis. For detailed guidelines, refer to **Performing the LR Library Transfer Reaction**, page 46.

Sequencing Entry Clones

Introduction

You may sequence entry clones generated by BP recombination using dye-labeled terminator chemistries including DYEnamic™ energy transfer or BigDye™ reaction chemistries.

Sequencing Primers

To sequence inserts in entry clones derived from BP recombination with pDONR™ 222, we recommend using the following sequencing primers. Refer to the following page for the location of the primer binding sites.

Forward primer (proximal to *att*L1)	M13 Forward (–20): 5′-GTAAAACGACGGCCAG-3′
Reverse primer (proximal to *att*L2)	M13 Reverse: 5′-CAGGAAACAGCTATGAC-3′

The M13 Forward (–20) and M13 Reverse Primers (Cat. nos. N520-02 and N530-02, respectively) are available separately from Invitrogen. For other primers, Invitrogen offers a custom primer synthesis service. For more information, visit our website (www.invitrogen.com) or contact Technical Support (page 54).

Note: If you experience difficulty using the M13 Reverse Primer to sequence entry clones, we recommend using an alternative reverse primer that hybridizes to the poly A tail of your cDNA insert. Design your reverse primer such that it is 5′-$(T)_{23}$N-3′ where N is A, C, or G.

General Guidelines

The AT rich *att*L sites in the entry clones may decrease the efficiency of the sequencing reactions. To optimize your sequencing reactions, we recommend the following:

- Plasmid DNA sample should be of good quality and purity (OD_{260}/OD_{280} = 1.7–1.99)
- During plasmid preparation, elute plasmid using deionized water instead of TE buffer

Sequencing Using BigDye™ Chemistry

To sequence entry clones using the BigDye™ chemistry, we recommend the following:

- Dilute plasmid DNA with deionized water to a final concentration of 100 ng/µL
- Use at least 700 ng of DNA
- Use 3.2 pmoles of primers
- Follow PCR conditions as specified in the BigDye™ sequencing kit

continued on next page

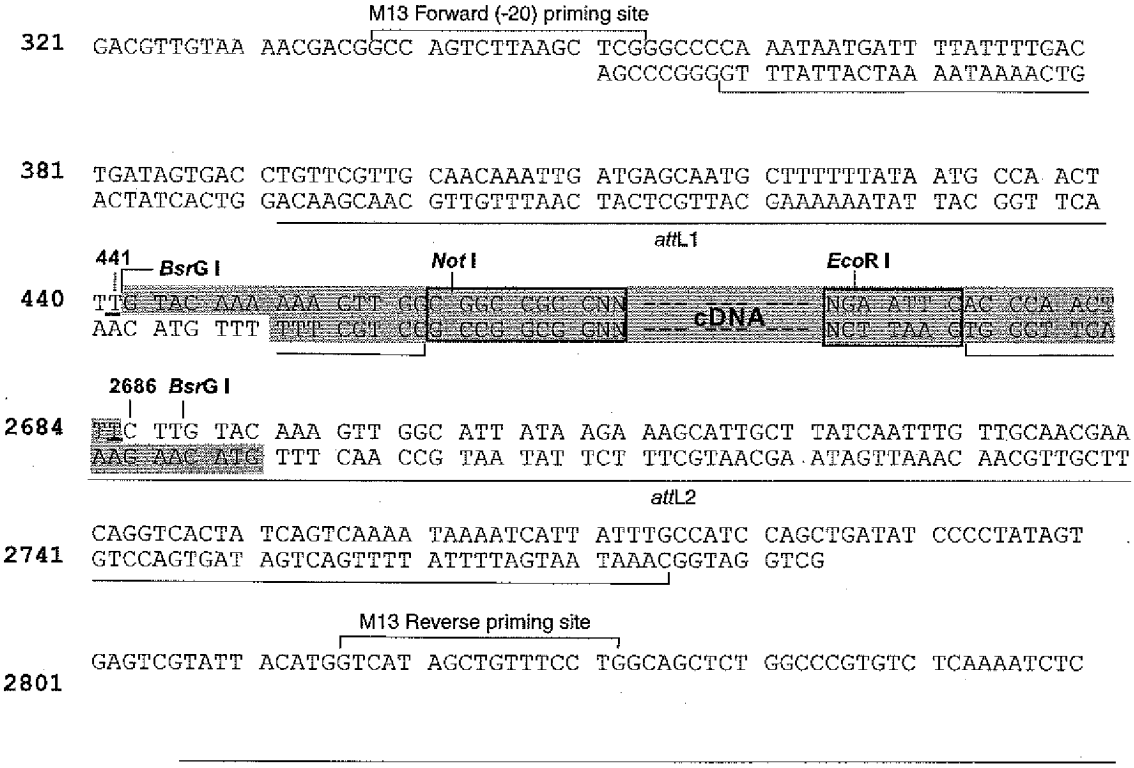
Sequencing Entry Clones, continued

Recombination Region

The recombination region of the entry library resulting from pDONR™ 222 × *att*B-flanked cDNA is shown below.

Features of the Recombination Region:

- Restriction sites are labeled to indicate the actual cleavage site.
- Shaded regions correspond to those DNA sequences transferred from the *att*B-flanked cDNA into the pDONR™ 222 vector by recombination. Non-shaded regions are derived from the pDONR™ 222 vector.
- Boxed region corresponds to sequences introduced from *att*B primers.
- Bases 441 and 2686 of the pDONR™ 222 sequence are marked.



Troubleshooting

Introduction

The following table lists some potential problems and possible solutions that may help you troubleshoot various steps during cDNA library construction. Note that the starting mRNA quality is a key factor that will affect the outcome of your results.

Problem	Cause	Solution
Low cDNA library titer (pUC19 transformation control working properly)	Insufficient starting mRNA	Quantitate the mRNA by measuring the A_{260}, if possible. We recommend using 5–10 µg of starting mRNA.
	Poorly prepared mRNA or degraded mRNA	• Follow the recommendations for mRNA isolation and working with mRNA (see page 7). • Resuspend RNA in DEPC-treated water. • Use the FastTrack™ MAG Micro mRNA Isolation Kit (Invitrogen Cat. no. K1580-02) for mRNA isolation.
	Insufficient cDNA yield for BP recombination reaction	• Perform BP reaction and construct a library from the contents of the column fraction in Tube 3 (page 26). • Make sure enzymes used in reaction are kept at the appropriate temperatures. • Maximize the yield during precipitation and fractionation. • Check cDNA recovery after size fractionation by Bioanalyzer.
	Poor electroporation efficiency	• ElectroMAX™ DH10B™ should not be thawed for longer than 20 min on ice. • Verify electroporator settings. If using electroporators other than those mentioned on page 33, optimize the settings using a control plasmid to $>1 \times 10^{10}$ cfu/µg DNA.

continued on next page

Troubleshooting, continued

Problem	Cause	Solution
Low cDNA library titer, continued	BP Clonase™ II enzyme mix inactive	• Perform positive control reaction to verify activity of enzyme mix. • Test a different aliquot of BP Clonase™ II enzyme mix. • Store BP Clonase™ II at –20°C in a frost-free freezer.
	Recombination reactions were not treated with proteinase K	Treat reactions with proteinase K before transformation.
Low percentage of full-length clones or low average insert size	mRNA sample partially degraded	• Follow the recommendations for mRNA isolation and working with mRNA (see page 7). • Always resuspend RNA in DEPC-treated water. • Use the FastTrack™ MAG Micro mRNA Isolation Kit (Invitrogen Cat. no. K1580-02) for mRNA isolation.
	Problem with size fractionation	Use lower volume for elution from Tube 2 (see page 26).
	Not enough starting mRNA	Use more material for library construction.
Few or no colonies obtained from the pUC19 transformation control	ElectroMAX™ DH10B™ competent cells stored incorrectly	Store competent cells at –80°C.
	Loss of transformation efficiency due to repeated freeze/thawing	After a tube of competent cells has been thawed, discard any unused cells.
	Transformation performed incorrectly	Follow the electroporation protocol for ElectroMAX™ DH10B™ competent cells on page 34. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Electroporator parameters not optimized	Verify electroporator settings. If using electroporators other than those mentioned on page 33, optimize the settings using a control plasmid to >1 × 10^{10} cfu/µg DNA.
	Loss of transformation efficiency due to arcing	See recommendations on page 33-34 to reduce chances of arcing.

continued on next page

Troubleshooting, continued

Problem	Cause	Solution
Low percentage of recombinants	Insufficient amount of cDNA used in the BP recombination reaction	Use the minimum amount of cDNA required for the BP recombination reaction. Use reduced amounts of adapter and primers for the BP recombination.
	Contamination from primers or adapters	• Wash the size fractionation column with enough volume before loading the sample, and make sure that it is not dried out. • Make sure there are no bubbles in the size fractionation column. • Elute the cDNA sample with a lower volume (e.g., 200 µL for Tube 2, and 40 µL for Tube 3.
	Non-specific recombination or deletion due leaky expression of proteins from cloned cDNA in bacteria.	• Prepare plasmid DNA from the low percentage library and run uncut plasmid DNA on a 0.8% low melt agarose gel to separate plasmids with deletions from plasmids with inserts. Excise gel band with the large sized cDNA and perform gel purification. Retransform purified plasmid DNA. • Repeat library construcion.
Low cDNA yield	Inaccurate incubation temperatures or temperature fluctuations	Perform the first strand reaction starting at 45°C. Keep reactions at 45°C when adding SuperScript® III RT.
	SuperScript® III RT inactive	• Store SuperScript® III RT at –20°C in a frost-free freezer. • Verify enzyme has not expired.
Low cDNA yield after size fractionation	Faulty columns	Check each column to verify that it is working properly. See page 25 for column specifications.
	Samples run too quickly over columns	Let columns drain completely before adding additional buffer.

Appendix

Accessory Products

Additional Products

Many of the reagents supplied with the Superscript® Full Length cDNA Library Construction Kit as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below. For more information, refer to our website (www.invitrogen.com) or contact Technical Support (page 54).

Item	Quantity	Catalog no.
SuperScript® III Reverse Transcriptase	2,000 units	18080-093
	10,000 units	18080-044
	4 × 10,000 units	18080-085
BP Clonase™ II Enzyme Mix	20 reactions	11789-020
	100 reactions	11789-100
LR Clonase™ II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-043
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	5 × 100 µL	12033-015
cDNA Size Fractionation Columns	3 columns	18092-015
Platinum® *Taq* DNA Polymerase High Fidelity	100 reactions	11304-011
Magna-Sep™ Magnetic Particle Separator	1 magnetic rack	K1585-01
T4 DNA Ligase	100 units	15224-017
RNase I (100 U/µL)	100 units	AM2294
DEPC-treated Water	4 × 1.25 mL	10813-012
FastTrack™ MAG Micro mRNA Isolation Kit	12 reactions	K1580-01
FastTrack™ MAG Maxi mRNA Isolation Kit	6 reactions	K1580-02
PureLink™ Quick Plasmid MiniPrep Kit	50 reactions	K2100-10
PureLink™ HiPure Filter Plasmid MidiPrep Kit	25 reactions	K2100-14
Kanamycin Sulfate (10 mg/mL)	100 mL	15160-054
RNase Away™ Reagent	250 mL	10328-011
RNaseZap®	250 mL	AM9780
E-Gel® EX Gel, 1%	10-Pak	G4010-01
E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit	1 kit	G6465
5X Second Strand Buffer	0.5 mL	10812-014

Gateway® Destination Vectors

A large selection of Gateway® destination vectors is available from Invitrogen to facilitate expression of your cDNA library in virtually any protein expression system. For more information about the vectors available and their features, refer to our website (www.invitrogen.com) or contact Technical Support (page 54).

Performing the LR Library Transfer Reaction

Introduction

Once you have qualified your cDNA library and analyzed entry clones, you can perform the LR recombination reaction to transfer your cDNA library into any Gateway® destination vector of choice. If you will be creating an expression library, you will need to follow the guidelines provided in this section for preparing DNA and for performing the LR recombination reaction.

Alternatively, you may screen your cDNA library to identify a specific entry clone and use this entry clone in an LR recombination reaction with a destination vector to generate an expression clone. Refer to the Gateway® Technology manual to perform a standard LR recombination reaction using a single entry clone.

Materials Required

Supplied by user:

- PureLink™ HiPure MidiPrep Kit, recommended (Invitrogen, Cat. no. K2100-14)
- LB media containing 50 µg/mL kanamycin
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)
- Your cDNA library
- Destination vector of choice (150 ng/µL)
- LR Clonase™ II enzyme mix (Invitrogen Cat. no. 11791-020)
- Ice bucket
- Proteinase K (2 µg/µL) (supplied with LR Clonase™ II enzyme mix)
- Sterile water
- Glycogen (20 µg/µL)
- 7.5 M NH_4OAc
- 100% ethanol
- Dry ice or a –80°C freezer
- 70% ethanol
- ElectroMAX™ DH10B™ T1 Phage Resistant Cells or equivalent

Preparing Double-Stranded DNA

You may prepare plasmid DNA from your cDNA library using your method of choice. We recommend using the PureLink™ MidiPrep Kit (Cat. no. K2100-14). Consider the following points when preparing your DNA:

- Inoculate 1×10^6–1×10^7 cfu of your cDNA library into 50 mL of LB containing 50 µg/mL kanamycin
- Grow the culture to an OD_{600} of 1.0 (approximately 8 hours)
- Use TE buffer, pH 8.0 to elute your DNA

continued on next page

Performing the LR Library Transfer Reaction, continued

Determining the DNA Yield

Use a NanoDrop or Bioanalyzer to check the yield of your DNA, or calculate the yield using A_{260} absorbance:

1. Measure the concentration of the plasmid DNA.
2. Determine the concentration using the equation below if measuring with a spectrophotometer at 260 nm:

   [DNA] = (A_{260}) (0.05 mg/mL) (dilution factor)
3. Determine the total yield by multiplying the concentration by DNA volume.
4. Dilute 100 ng of the DNA to 25 ng/µL.
5. If the concentration is <25 ng/µL perform **Ethanol Precipitation** (see below).

Ethanol Precipitation

1. Add reagents to your plasmid DNA sample in the following order:

Reagent	Amount	
Glycogen (20 µg/µL)	1 µL	
7.5 M NH_4OAc	0.5 volume	(*i.e.* 0.5 × volume of cDNA)
100% ethanol	2.5 volumes	[*i.e.* 2.5 × (volume of cDNA + NH_4OAc)]

2. Place the tube in dry ice or at –80°C for at least 1 hour. Centrifuge the sample at +4°C for 30 minutes at 16,000 × g.
3. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150 µL of 70% ethanol.
4. Centrifuge the sample at +4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.
5. Dry the cDNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.
6. Resuspend the cDNA pellet in 5–10 µL of TE buffer.

LR Library Transfer Reaction

If you have a positive control plasmid for the LR recombination reaction, we recommend including it in your experiment to help you evaluate your results.

1. Add the following components to a sterile 1.5 mL microcentrifuge tube at room temperature and mix.

Component	Sample	Negative Control	Positive Control
cDNA entry library (25 ng/µL)	2 µL	--	--
Positive control plasmid (25 ng/µL)	--	--	2 µL
Destination vector (150 ng/µL)	3 µL	3 µL	3 µL
TE Buffer, pH 8.0	9 µL	11 µL	9 µL
Total volume	**14 µL**	**14 µL**	**14 µL**

continued on next page

Performing the LR Library Transfer Reaction, continued

LR Library Transfer Reaction, continued

2. Remove the LR Clonase™ II enzyme mix from –20°C and thaw on ice (~2 minutes).
3. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. Add 6 µL of LR Clonase™ II enzyme mix to each sample. Mix well by vortexing briefly twice (2 seconds each time).

   Reminder: Return LR Clonase™ II enzyme mix to –20°C immediately after use.
5. Incubate reactions at 25°C for 16–20 hours.
6. Add 2 µL of the proteinase K solution to each reaction. Incubate the reactions at 37°C for 15 minutes, then at 75°C for 10 minutes.
7. Proceed to **Ethanol Precipitation**, below.

Ethanol Precipitation

1. To the LR reaction, add reagents in the following order. Be sure to use sterile water and not DEPC-treated water.

Sterile water	80 µL
Glycogen (20 µg/µL)	1 µL
7.5 M NH_4OAc	50 µL
100% ethanol	375 µL

   Note: You may stop at this point and store the tube at –20°C overnight if necessary.
2. Place tube in dry ice or at –80°C for 10 minutes. Centrifuge the sample at +4°C for 25 minutes at 16,000 × g.
3. Carefully remove the supernatant trying not to disturb the pellet. Add 150 µL of 70% ethanol.
4. Centrifuge the sample at +4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.
5. Dry the DNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.
6. Resuspend the DNA pellet in 9 µL of TE buffer by pipetting up and down 30–40 times.

Transforming Competent *E. coli*

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, DH10B™ or equivalent for transformation. **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F' episome (*e.g.* TOP10F'). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

We recommend using ElectroMAX™ DH10B™ T1 Phage Resistant Cells for maximum transformation efficiency. If you will be using ElectroMAX™ DH10B™ T1 Phage Resistant cells, follow the guidelines outlined in the section entitled **Transforming Competent Cells**, page 28.

continued on next page

Performing the LR Library Transfer Reaction, continued

Analyzing the Expression Library

Follow the guidelines outlined in the section entitled **Analyzing the cDNA Library**, page 36, to determine the titer, average insert size, and percent recombinants of your expression library. We recommend that you:

- Analyze transformants by digesting with *Bsr*G I which cuts within both *att*B sites of the expression library as well as within the *att*R sites and *ccd*B gene for non-recombined destination vectors
- Digest and electrophorese your destination vector with no insert to determine the background *Bsr*G I digestion pattern for your particular destination vector

What You Should See

When starting with ≥5 x 10^6 cfu from your cDNA entry library, you should obtain 5 x 10^6 – 1 x 10^7 primary clones from one LR recombination reaction. If the number of primary clones is considerably lower for your expression library, you may perform additional LR recombination reactions using any remaining plasmid DNA from your entry library.

The average insert size and percentage of recombinants of your expression library should be maintained from your cDNA entry library.

Examples of Results

Introduction

In this section, some examples are provided to illustrate titer determination and qualification of the cDNA library. All steps were performed according to the protocols in this manual.

Determining the cDNA Library Titer

The results of the plating assay are listed below.

Dilution	Amount Plated (µL)	Colonies Per Plate
10^{-2}	100 µL	654
10^{-3}	100 µL	54
10^{-4}	100 µL	7

The titer for each plate was determined using the results of the plating assay and the equation below. For the 10^2 dilution:

$$\text{cfu/mL} = \frac{\text{colonies on plate} \times \text{dilution factor}}{\text{volume plated (ml)}}$$

$$= \frac{654 \text{ colonies} \times 100}{0.10 \text{ ml}}$$

$$= 6.54 \times 10^5 \text{ cfu/mL}$$

The titer for each plate was used to calculate the average titer of the cDNA library.

The total colony-forming units was determined by multiplying the average titer by the total volume of the cDNA library. In this experiment, 6 electroporations were performed to result in a total volume of 12 mL.

The calculated titers and total number of colony-forming units are shown below.

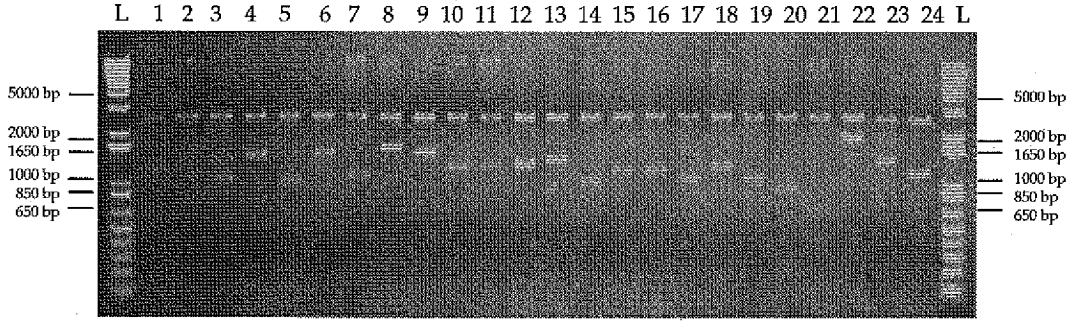
Dilution	Amount Plated (µL)	Colonies Per Plate	Titer (cfu/mL)	Average Titer (cfu/mL)	Total Volume (mL)	Total CFUs (cfu)
10^{-2}	100 µL	654	6.54×10^5			
10^{-3}	100 µL	54	5.4×10^5	6.31×10^5	12	7.6×10^6
10^{-4}	100 µL	7	7×10^5			

continued on next page

Examples of Results, continued

Qualifying the cDNA Library

Plasmid DNA was isolated from 24 colonies using the PureLink™ Quick Plasmid MiniPrep Kit from Invitrogen. 300–500 ng of plasmid DNA and 250 ng of supercoiled pDONR™ 222 were digested with *Bsr*G I and run on a 1% agarose gel stained with ethidium bromide. Results are shown below. Note that pDONR™ 222 (lane C) gives a digestion pattern of 2.5 kb, 1.4 kb, and 790 bp when digested with *Bsr*G I.



L = 1 Kb DNA Plus Ladder from Invitrogen
C = Digested pDONR™ 222

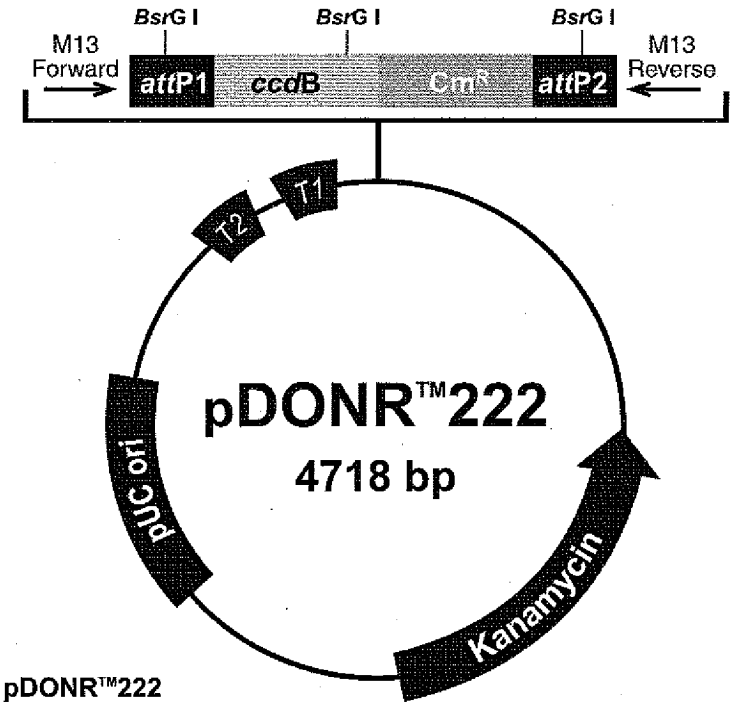
Clone	Band Size (kb)	Insert Size (kb)	Clone	Band Size (kb)	Insert Size (kb)
1	1.5 + 1.3	2.8	13	1.4	1.4
2	0.8	0.8	14	1.0	1.0
3	1.2	1.2	15	1.2	1.2
4	1.6	1.6	16	1.2	1.2
5	1.1	1.1	17	1.1	1.1
6	1.6	1.6	18	1.5	1.5
7	1.2	1.2	19	1.1	1.1
8	1.8	1.8	20	1.1	1.1
9	1.6	1.6	21	0.8 + 0.5	1.3
10	1.3	1.3	22	2.2	2.2
11	1.3	1.3	23	1.5	1.5
12	1.3	1.3	24	1.3	1.3

Number of Clones Analyzed	24
Number of Clones Containing Inserts	24
Percent Recombinants	100%
Average Insert Size (kb)	1.4
Insert Size Range (kb)	0.8–2.8

Map and Features of pDONR™ 222

pDONR™ 222 Map

The map below shows the elements of pDONR™ 222. The complete sequence of pDONR™ 222 is available from our website (www.invitrogen.com) or by contacting Technical Support (page 54). See page 54 for a map of the vector after the BP recombination reaction.



Comments for pDONR™222
4718 nucleotides

*rrn*B T2 transcription termination sequence: bases 58-85 (c)
rrnB T1 transcription termination sequence: bases 217-260 (c)
M13 Forward (-20) priming site: bases 327-342
*att*P1: bases 360-591
*Bsr*G I restriction sites: bases 442, 1232, 2689
*ccd*B gene: bases 987-1292 (c)
Chloramphenicol resistance gene: bases 1612-2295 (c)
*att*P2: bases 2543-2774 (c)
M13 Reverse priming site: bases 2816-2832
Kanamycin resistance gene: bases 2899-3714 (c)
pUC origin: bases 4045-4718

(c) = complementary strand

continued on next page

Map and Features of pDONR™ 222, continued

Features of the Vector

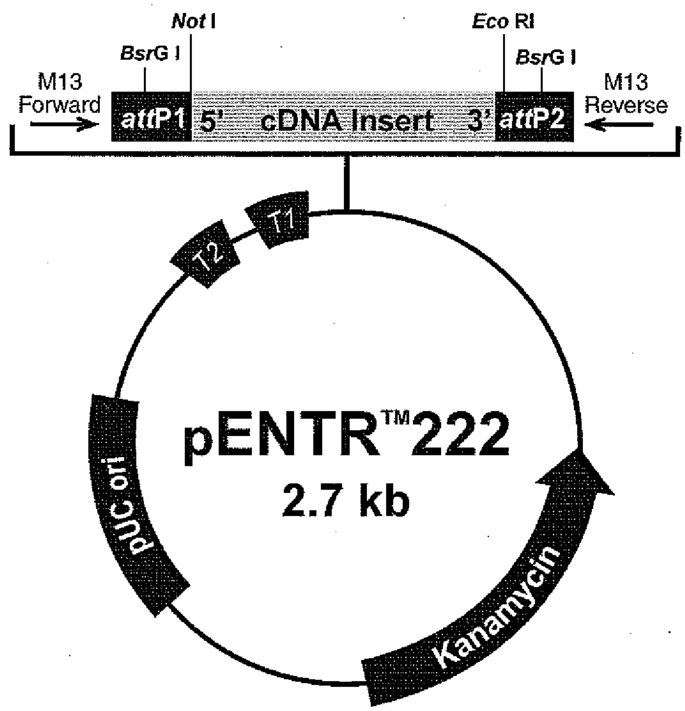
pDONR™ 222 (4718 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription terminators	Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz *et al.*, 1991).
M13 forward (–20) priming site	Allows sequencing in the sense orientation.
*att*P1 and *att*P2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of *att*B-containing cDNA (Landy, 1989).
*Bsr*G I restriction sites	Allows detection and size determination of cDNA inserts by restriction enzyme analysis.
*ccd*B gene	Allows negative selection of the plasmid.
Chloramphenicol resistance gene	Allows counterselection of the plasmid.
M13 reverse priming site	Allows sequencing in the anti-sense orientation.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli.*
pUC origin	Allows high-copy replication and maintenance of the plasmid in *E. coli.*

Map and Features of pENTR™ 222

pENTR™ 222 Map

The map below shows the elements of pENTR™ 222. See page 41 for information on the sequence flanking your cDNA insert.



pENTR™ 222 MCS

```
                          M13 Forward (-20) priming site
321   GACGTTGTAA AACGACGGCC AGTCTTAAGC TCGGGCCCCA AATAATGATT TTATTTTGAC
                                       AGCCCGGGGT TTATTACTAA AATAAAACTG

381   TGATAGTGAC CTGTTCGTTG CAACAAATTG ATGAGCAATG CTTTTTTATA ATG CCA ACT
      ACTATCACTG GACAAGCAAC GTTGTTTAAC TACTCGTTAC GAAAAAATAT TAC GGT TCA
                                          attL1
      441 BsrG I                 Not I                          EcoR I
440   TTG TAC AAA AAA GTT GGC GGC CGC CNN --- cDNA --- NGA ATT CAC CCA ACT
      AAC ATG TTT TTT CGT CCG CCG GCG GNN ---      --- NCT TAA GTG GGT TGA

      2686 BsrG I
2684  TTC TTG TAC AAA GTT GGC ATT ATA AGA AAGCATTGCT TATCAATTTG TTGCAACGAA
      AAG AAC ATG TTT CAA CCG TAA TAT TCT TTCGTAACGA ATAGTTAAAC AACGTTGCTT
                                          attL2
      CAGGTCACTA TCAGTCAAAA TAAAATCATT ATTTGCCATC CAGCTGATAT CCCCTATAGT
2741  GTCCAGTGAT AGTCAGTTTT ATTTTAGTAA TAAACGGTAG GTCG

                          M13 Reverse priming site
      GAGTCGTATT ACATGGTCAT AGCTGTTTCC TGGCAGCTCT GGCCCGTGTC TCAAAATCTC
2801
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Technical Support

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